University of Alberta

Bacteriocin Production and Regulation in *Carnobacterium piscicola* LV17

by

Linda Saucier ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Microbiology

Edmonton, Alberta

Spring 1997

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Jan. 21, 97

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Bacteriocin Production and regulation in *Carnobacterium piscicola* LV17 submitted by Linda Saucier in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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A la mémoire de mes grands-mères Blanche et Yvonne, dont les prières de l'une et le courage de l'autre ont su inspirer chacune de mes journées.

ABSTRACT

Carnobacterium piscicola LV17 is a non-aciduric lactic acid bacterium that was isolated from vacuum packaged pork. It produces 3 bacteriocins which could be of special interest in biopreservation of food because they are active against strains of *Listeria* and *Enterococcus* and production occurs early in the growth cycle when inoculated at 10^7 cfu/ml. Competitive studies between *C. piscicola* LV17 and *Carnobacterium divergens* LV13 were done to evaluate the inoculum size of *C. piscicola* LV17 necessary to inhibit the growth of the target organism. These studies revealed that bacteriocin production in *C. piscicola* LV17 is inoculum dependent. Bacteriocin production in the supernatant was detected only when the producer was inoculated at $\geq 10^6$ cfu/ml. The bacteriocin negative phenotype was only temporarily lost and could be recovered by growth on solid medium or by the addition (1%) of cell free supernatant from a carnobacteriocin-producing culture prior to the stationary phase of growth. Bacteriocin induction by addition of the purified bacteriocins or their sulfoxide derivatives indicated that bacteriocin production in *C. piscicola* LV17 is an autoregulated trait. Bacteriocin regulation was investigated at the molecular level. Northern blot analysis of *cbnA*, *cbnB2* and *cbnBM1* indicated that bacteriocin production from a high inoculum and bacteriocin induction are controlled at the transcriptional level. Transcription was initiated in both cases from the same promoter located upstream of the structural gene for each bacteriocin. Bacteriocin production is observed in a meat system using a low inoculum ($\leq 10^4$ cfu/g). However, conditions such as pH of the meat, storage temperature, medium composition (i.e., source of yeast extract) and inoculation procedures influence the efficiency of bacteriocin production. Bacteriocin production is not a constitutive trait in *C. piscicola* LV17

but it is regulated under specific conditions which could be affected by the habitat, such as the surface of meat. A model is proposed to explain how bacteriocin production is induced at high cell density and autoregulated in *C. piscicola* LV17.

ACKNOWLEDGMENTS

First of all, I would like to thank my supervisor, Dr. M.E. Stiles, for giving me the opportunity to come to his laboratory to do my Ph.D. I learned a lot with his group. Special thanks are addressed to Ms. Alison Poon and Dr. Lynn Elmes who helped me on a daily basis. My work was eased by the collaboration of several faculty members of different Departments. Dr. John C. Vederas and Dr. Mila Sailer from the Department of Chemistry who supplied me with the pure bacteriocins. Dr. Susan E. Jensen and Dr. Ashish S. Paradkar have played a major role in the accomplishment of the RNA work and to my introduction to genetics. Special thanks go to Dr. Laura S. Frost. She gave me access to equipment and materials from her laboratory which substantially eased my work. Also from Dr. Frost's laboratory, I express sincere thanks to Mrs. Lori J. Jerome who helped solving my Northern nightmares and Ms. Karen G. Antony who guided me with the sequencing and primer extension experiment. Dr. Gordon G. Greer was involved in the applied aspect of my work. Many thanks for his help and support. I also extend my thanks to Mr. Bryan D. Dilts for his technical support. My study was funded by Agriculture and Agri-Food Canada and I am deeply thankful for their faith in me and especially for the support of Dr. Wayne Modler, Dr. Karen Lapsley, Dr. Claude Aubé and Dr. J.-P. Gabriel Piette.

There is no way that I would have been able to go through this journey without the unconditional support of my fiancé, Dr. Sylvain Moineau. The distance has never been an obstacle for his daily presence, especially in the darker days. I also thank my immediate and extended family who have always encouraged and cared for me.

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List of Abbreviations

AMV	avian myeloblastosis virus
APT	All Purpose Tween
AU	Activity Unit
Bac^-	bacteriocin negative
Bac^+	bacteriocin positive
bp	base pair(s)
Cbn	carnobacteriocin(s)
cfu	colony forming units
CMM	Cooked Meat Medium
cpm	counts per minute
EDTA	ethylenediaminetetraacetic acid
IR	inverted repeat(s)
kb	kilobase(s)
LAB	lactic acid bacteria
Lcn	leucocin A-UAL187
M	methionine
MAP	modified atmosphere packaging
MW	molecular weight
ND	not determined
neg	negative
Nis	nisin
OD_{600}	optical density at 600 nm
orf	open reading frame(s)

PAGE	polyacrylamide gel electrophoresis
Pln	plantaricin
r	resistant
RBS	ribosome binding site
s	sensitive
Sak	sakacin
SDS	sodium dodecyl sulphate
SSPE	sodium chloride/sodium phosphate/EDTA
TFA	Trifluoroacetic acid
UAL	University of Alberta culture collection
VP	vacuum packaged

Ora et Labora

Chapter 1

General Introduction

1.1. Importance of the meat industry in the Canadian economy

In the highlights of the report of the Canadian Meat Council in January 1995, it was stated that in 1992 the meat and meat products industry was the fourth most important manufacturing industry in Canada. In 1993, the meat and meat products industry (excluding poultry) had the highest food commodity sales, totaling $9.2 billion, followed by dairy products with $7.3 billion, fruit and vegetables at $3.4 billion, and bakery products, fish and poultry with approximately $2.5 billion each.

In 1995, the total exports of pork and beef were estimated at $1.6 billion. The challenge for the Canadian meat industry in the coming years is to reduce live animal exports, keeping them in Canada for the production of value-added products and thus creating jobs within our borders. Access to distant countries is limited by the shelf life of the meat products. Freezing technology is available but it has the price associated with it that makes it difficult to compete with locally-manufactured products. New technology that will allow us to access distant markets at a better price will contribute to the development of our exports as value-added products. Furthermore, consumers request foods that are as "natural" as possible with little or no processing (Miller Jones, 1992; Rhodehamel, 1992; Gould, 1995; Roller, 1995).

Even if our meat industry is flourishing, losses due to spoilage and as a result of illness due to meat consumption are likely to affect the profit margin of the industry. These losses include the cost of medical fees, loss of income (on the part of both company and victim), legal awards and settlements, loss of leisure time, pain, grief, suffering and death, epidemiological investigation and

laboratory costs. A large part of that bill is paid by the food supplier (Todd, 1989).

Gravani (1987) listed the most common characteristics of foods implicated in foodborne disease. Implicated foods provided nutrients in sufficient quantity and variety to support microbial growth, water activity was above 0.85 and pH was greater than 4.6. Also, storage temperature and available oxygen allowed the growth of the causative organism. All of these characteristics are present in meat and its storage conditions, and one-third of the foods incriminated in foodborne disease in North America are of meat origin (Todd 1992, 1994).

From 1975 to 1987, foodborne diseases of known etiology were mostly of bacterial origin (Todd 1992, 1994). The pathogenic microorganisms representing the greatest risk with meat-and poultry-borne disease are *Salmonella* spp., *Campylobacter* spp., verotoxigenic *Escherichia coli* (VTEC), *Listeria monocytogenes* and *Toxoplasma gondii* (Todd and Harwig, 1996). Listeriosis has been associated with meats in several countries but not in Canada (Todd and Harwig, 1996). However, *L. monocytogenes* has been identified as an emerging foodborne pathogen in chilled foods and could present a threat in food where low temperature is the only hurdle against microbial growth, especially in so-called "ready-to-eat meals" (Schofield, 1992).

1.2. Food preservation

1.2.1. Brief overview of the history of food preservation

The safety and preservation of our food supply was, is and will always be a concern. As early as 800 000 BC, our prehistoric ancestors realized that meat and fish preserved better when they were cooked. So the use of fire or reduction of microbial load by heat treatment was man's first means of food preservation. The first oven was a simple structure: a hole with hot stones. It was only in the nineteenth century that we developed the ability to use thermal preservation to achieve an appreciably longer shelf life. This resulted from the work of Nicolas Appert (1749-1841) on "appertization" or cooking in a tight container, now referred to as canning or sterilization (Toussaint-Samat, 1994). Salt has been applied for both preservation and as a flavoring agent since ancient times. As a preservative salt reduces the a_w (water activity, a measure of the available water for microbial growth).

Ancient methods of food preservation also included drying and smoking either by using the sun as a source of energy or by fire, the latter added the preservative effect of formaldehyde, phenols and organic acids present in the smoke (Maga, 1988). Masson and Challet in 1975 were the first to artificially dry vegetables in a hot-air room (Ray, 1992). In the late 1950s, a new process called freeze drying (lyophilization or cryophilization) allowed the dehydration of food with reduced heat and oxidation damage. However, because of its cost, the use of this process is still limited to high value products (Thorne, 1986).

Spices and nitrite were used in the Middle Ages for preservation and enhancing the sensory attributes of foods (Miller Jones, 1992). Despite

irrefutable demonstrations of their antimicrobial properties in laboratory cultures, spices are less inhibitory in food because high concentrations are needed to achieve preservation. Among the gram-positive bacteria, lactic acid bacteria are most resistant to these antimicrobials (Jay, 1992; Roller, 1995). Prior to use of ionizing radiation to sterilize spices, they were commonly contaminated with bacteria, yeast, molds and spores (Jay, 1992) all of which can affect the preservation of foods. Considering the fact that sensory evaluation was probably the only means of testing the efficiency of spices as preservatives, it is likely that their positive effect was mainly due to masking of the putrid characteristics of the food, as suggested by Thorne (1986): "Kitchens at the time were often stinking and strong herbs were used to disguise the stench of putrid meats".

Only the Eskimos were able to make do without large quantities of salt, because they could rely on the cold weather for food preservation. Ferdinand Carré (1824-1900) and Charles Tellier (1828-1913) are considered the fathers of refrigeration; however, the freezing techniques used by Carré and Tellier produced meat that, once thawed, was spongy and not good for roasting. In their case, the freezing was slow, allowing the formation of large water crystals that broke the cell structure of the food. Clarence Birdseye in 1929 developed Quick-Freezing or "surgelé" technology. Visiting the Eskimos of Labrador, he realized that the speed at which the food is frozen makes a major difference to the final quality of the frozen food (Toussaint-Samat, 1994).

Although Egyptians in ancient times had already mastered the preservation of large quantities of grain between harvests, the first large scale food industry was beer brewing (Thorne, 1986). Fermentation has been used

as a form of food preservation since biblical times. It is now known that antimicrobial activity of the fermenting microflora is responsible for this effect (Table 1-1). The original fermented drink was mead, a naturally fermented mixture of honey and water (Toussaint-Samat, 1994). Other references indicate that fermented foods have been consumed for the past 8000 years (Ray, 1992).

Pasteur finally disproved the theory of spontaneous generation and he provided the foundation for our knowledge of food microbiology (Thorne, 1986). A sound food fermentation industry could not have relied on an opportunistic process. The knowledge of the microbial world and the development of starter cultures at the end of the 1800's secured a sustainable industry by assuring a sustainable product quality and a reliable process which have survived the challenge of time (Crawford, 1958).

1.2.2. Modern perspective of biopreservation

As highlighted in the previous section, we now have technologies that allow us to access different kinds of food throughout the year. Table 1-2 lists some of the newer methods of food preservation that are currently under investigation. Despite scientific innovations, consumer demands greatly influence the type of food that we find in the supermarket. To a certain extent, consumer choices can be influenced by health facts and convenience. The best example to illustrate this is the consumer resistance to the use of irradiation as a method of food preservation.

Today consumers demand foods that are minimally processed, as

Table 1-1. Inhibitory compounds produced by microorganisms [a]

Nature of compounds	Examples
Organic acids	acetic propionic lactic
By-product of pyruvate metabolism	diacetyl
Electron acceptor	hydrogen peroxide
Lytic enzymes	glucanase
Peptides	bacteriocins
By-product of glycerol metabolism	reuterin
Gas	CO_2
Alcohol	ethanol

[a] Adapted from Ray and Daeschel (1992) and Roller (1995).

Table 1-2. Newer methods for food preservation [a]

Methods	Description
Physical	
Radurization, radicidation, radappertization	application of ionizing irradiation
Pressurization	application of high hydrostatic pressure
"Electroporation"	application of high electric discharges
Non-coherent light pulse	application of high intensity laser
Modified atmosphere packaging	modification of gas phase to inhibit growth of targeted organisms
Non physical	
lysozyme, glucanase, lactoperoxidase	bactericidal enzymes
lactoferrin, lactoferricin	iron chelators
bacteriocins	membrane active peptides (pore formers)

[a] Adapted from Gould (1992 and 1995).

"natural" as possible, yet remain convenient to use (Miller Jones, 1992; Rhodehamel, 1992; Gould, 1995; Roller, 1995). Refrigeration is the technology that will most likely fulfill this demand; however, psychrotrophic and psychrophilic microorganisms that are able to grow at refrigeration temperatures have raised public health concerns and can cause economic losses due to spoilage (Schofield, 1992).

Current recommendations to insure the control of psychrotrophic and psychrophilic microorganisms have included the implementation of Hazard Analysis Critical Control Point (HACCP) and incorporation of multiple "challenge" hurdles (such as preservatives and inherent characteristics of the food) to control these organisms in food manufacturing (Ray, 1992; Gould, 1995). In the meat industry, modified atmosphere packaging (MAP) is used to extend the shelf life of meats. When the meats are packaged under anaerobic conditions, a different microflora develops. A putrefactive flora develops when the meat is stored under refrigeration in aerobic packaging; the shelf life obtained in such conditions is limited to a few days. When the meat is stored in anaerobic conditions using modified atmosphere packaging, facultative anaerobic lactic acid bacteria grow and the shelf life is extended to several weeks (Dainty and Mackey, 1992). This increase in shelf life is achieved provided that non-spoiling lactic acid bacteria grow and become part of the prevailing microflora. Several laboratories around the world are searching for suitable lactic acid bacteria that could act as protective cultures in meats. The discovery that many lactic acid bacteria produce antimicrobial peptides, referred to as bacteriocins, has stimulated this field of research. In a study on cooked pork, Fang and Lin (1994) demonstrated that using a combination of hurdles consisting of modified atmosphere, nisin and low temperature, growth

of both the spoilage bacteria *Pseudomonas fragi* and the psychrotrophic pathogen *L. monocytogenes* was reduced. The modified atmosphere conditions with elevated concentration of CO_2 were inhibitory for *P. fragi*, whereas nisin was the hurdle that affected the growth of *L. monocytogenes*. Furthermore, the synergistic effect obtained by simultaneous utilization of multiple bacteriocins and the fact that some bacteriocins have more activity in the temperature range of 4 to 15°C reinforces the potential of bacteriocinogenic lactic microflora as a hurdle for meat products stored under refrigeration conditions (Roller, 1995).

1.3. Bacterial succession in meat

The chemical composition of the lean muscles of poultry, fish, and food animals is 74 to 80% water, 15 to 22% protein, 0.7 to 4.3% lipids and 1.2 to 3.5% low molecular weight components that include: nucleosides, nucleotides, free amino acids, lactic acid, glycogen, glucose and glucose-6-phosphate (Greer, 1989). These low molecular weight compounds are readily available to sustain growth of the indigenous microflora to 10^9 bacteria per gram. Proteins are not degraded until the low molecular weight compounds have been exhausted, by that time the meat is spoiled, indicating that proteolysis is a post-spoilage phenomenon (Greer, 1989). Presence of the same low molecular weight compounds on adipose tissue allows bacterial growth and, like proteolysis, lipolysis is a post-spoilage phenomenon (Greer, 1989). The more neutral pH of fat tissue favors growth of certain organisms compared with lean muscle i.e., *Hafnia alvei*, *Serratia liquefaciens*, *Lactobacillus plantarum*, *Alteromonas putrefaciens*, and *Brochothrix thermosphacta* (Greer, 1989).

Meat carcasses can contain up to 10^4 bacteria/cm^2 after dressing (Dainty and Mackey, 1992; Gill and Bryant, 1992). The initial microflora is mainly mesophilic and located on the surface of the meat because the edible tissue of a healthy animal is either sterile or contains very low microbial numbers (Urbain and Campbell, 1987; Dainty and Mackey, 1992). The surface of the carcass is contaminated by organisms from the hide or skin of the animal, gut contents, workers' hands and the slaughterhouse environment (Dainty and Mackey, 1992). Shelf life of meat is affected by various factors such as storage temperature, gas composition of the head space of the package, indigenous enzymes, type of meat, level of dehydration of carcasses, chilling and microbial growth, the latter being by far the most important (Lambert *et al.*, 1991d).

The prevailing microflora on meat varies with storage conditions (Table 1-3). Under refrigeration a psychrotrophic flora prevails. Several different genera grow on aerobically packaged meat (Table 1-3). Because of a better growth rate than the other microorganisms under aerobic conditions, *Pseudomonas* spp. prevail and constitute up to 50 to 90% of the overall microbial population. *Enterobacteriaceae* prevail under conditions of poor refrigeration (10°C) and spoil the meat (Gill and Newton, 1978; Dainty and Mackey, 1992; Stiles, 1991b). When the meat is placed in an anaerobic environment, a lactic microflora prevails because they are more tolerant of CO_2 than the pseudomonads and the *Enterobacteriaceae*. CO_2 affects microbial growth by extending the lag phase and increasing the generation time. Several mechanisms have been proposed to explain the inhibitory effect of CO_2: displacement of O_2, decrease of pH, interference with metabolic enzymes and membrane stability (Daniels *et al.*, 1985; Stiles, 1991b). Using an

Table 1-3. Original microflora of meat and differences in their development with optimal chilled storage conditions [a]

Initial microflora (After dressing)	Aerobic storage	Anaerobic storage
micrococci	*Pseudomonas*	*Lactobacillus*
staphylococci	*fragi*	*sake*
Bacillus spp.	*lundensis*	*curvatus*
coryneforms	*fluorescens*	*"bavaricus"*
Enterobacteriaceae	*Psychrobacter*	*Carnobacterium*
"flavobacteria"	*immobilis*	*divergens*
pseudomonads	*Acinetobacter*	*piscicola*
lactic acid bacteria	*Shewanella*	*Leuconostoc*
Brochothrix	*putrefaciens*	*carnosum*
themosphacta	*Brochothrix*	*gelidum*
	thermosphacta	*mesenteroides*
	lactic acid bacteria	ssp. *mesenteroides*

[a] Adapted from Dainty and Mackey (1992) and Greer (1989).

atmosphere of 100% N_2 it was demonstrated that the effect of CO_2 goes beyond simple displacement of O_2 as a mixture of N_2 and CO_2 is more inhibitory (Daniels *et al.*, 1985; Stiles, 1991b). In vacuum packaged meat, the residual O_2 is rapidly used up through muscle and microbial respiration and a concentration of 10-20% to a maximum of 30% of CO_2 can be reached (Stiles, 1991b). Several factors influence the efficacy of CO_2: sensitivity of the target organism, concentration of CO_2 (a minimum of 20 to 30% is necessary, Stiles, 1991a), storage temperature (solubility increase at lower temperature) and time of application of CO_2 relative to growth phase of the target organism (CO_2 is more efficient prior to the growth phase; Stiles, 1991b; Lambert *et al.*, 1991d).

The extended storage life of meat under anaerobic conditions is due to the fact that the lactic acid bacteria that grow on meat cause spoilage only after maximum populations are reached, whereas the aerobic spoilage bacteria cause putrefactive odors earlier in the growth cycle. The defect caused by lactic acid bacteria is described as "souring" which is less offensive than the putrefaction that develops aerobically (Dainty and Mackey, 1992). However, some lactic acid bacteria, such as *Lactobacillus sake,* can cause spoilage by the production of hydrogen sulfide, reinforcing the necessity to select non-spoiling lactic acid bacteria for use as protective cultures (Stiles, 1991b, 1994). Furthermore, the prevalence of lactic acid bacteria exerts an inhibitory pressure on several foodborne pathogens and improves the safety of the product (Stiles, 1994).

This is what happens in meat at a normal pH of 5.5 to 5.7. If animals are subjected to stress before slaughter, the reserves of glycogen will be depleted and the pH will remain closer to physiological pH i.e., above pH 6.0. This

higher pH favors the growth of *Enterobacteriaceae, B. thermosphacta*, and the psychrotrophic pathogen *Yersinia enterocolitica* (Lambert *et al.*, 1991d; Dainty and Mackey, 1992); a similar phenomenon is observed on fat tissue because its pH is higher. Maximal O_2 exclusion is imperative to control these organisms on meat at high pH (Gill and Newton, 1978, Dainty and Mackey, 1992). *Enterobacteriaceae* cause spoilage by producing sulfury odors, probably due to the production of hydrogen sulfide; whereas *B. thermosphacta* gives dairy/butter/cheesy odors apparently due to acetoin, diacetyl, 3-methyl-1-butanol and 2-methyl propanol (Greer, 1989; Dainty and Mackey, 1992).

Safety of vacuum and MAP fresh meat was challenged when it was observed that *Clostridium* species can represent a significant portion of the prevailing microflora (Kalchayanand *et al.*, 1989) and when toxin production by *Clostridium botulinum* was observed on irradiated fresh pork packaged in MAP and stored at a temperature of 15°C (Lambert *et al.*, 1991a, b, c, d). *Listeria* strains were isolated in MAP fresh pork at -1°C but not at 4°C or 10°C (McMullen and Stiles, 1993). Because temperature can fluctuate during storage, it is not advisable to rely only on it to control these pathogens. Competitive inhibition using lactic acid bacteria has been proposed as an additional hurdle. Lactic acid bacteria strains for use as protective cultures should not spoil meat and should have a growth rate that allows them to dominate the meat microflora. Such protective cultures are not yet used commercially and research is still in progress to develop such strains.

1.4. What are lactic acid bacteria?

The lactic acid bacteria (LAB) refer to a group of gram-positive

microorganisms that produce lactic acid as the major end product of carbohydrate fermentation. They belong to the *Clostridium* branch of gram-positive bacteria with < 50% guanosine and cytosine (G+C) content (Axelsson, 1993; Pot *et al.*, 1994; Vandamme *et al.*, 1996). The genera that fit the description of the typical LAB are listed in Table 1-4. This definition excludes the genus *Bifidobacterium* because they produce 1 mole of lactic acid and 1.5 moles of acetic acid per mole of glucose from the bifidus pathway and their high mol% G+C (54.2 to 64.5%) content places them into the *Actinomyces* branch of the gram-positive eubacteria (Axelsson, 1993; Ballongue, 1993; Pot *et al.*, 1994; Vandamme *et al.*, 1996). However, because bifidobacteria are associated with fermented dairy products (e.g., yogurt) as part of the fermenting flora or as a post-fermentation adjunct with other "true" LAB (Ballongue, 1993), they are often described together. Morphologically, LAB are cocci, coccobacilli or rod-shaped bacteria (Vandamme *et al.*, 1996). Growth conditions and the stage of growth can influence cell morphology. For example, the coccobacillary shape of many heterofermentative lactobacilli makes it difficult to discriminate them from *Leuconostoc* spp. (Pot *et al.*, 1994).

LAB are fastidious chemoorganotrophic organisms that are strictly fermentative: many of them are homofermentative organisms but *Leuconostoc*, *Carnobacterium* and a subgroup of *Lactobacillus* are heterofermentative (Axelsson, 1993; Schleifer and Ludwig, 1995). LAB are associated with various foods such as milk, vegetables and muscle foods, and commercial starters are available to produce fermented foods and beverages (yogurt, malolactic fermentation in wine, sausage, sauerkraut, etc.). They also constitute part of the microflora of the mammalian mucosa (Axelsson, 1993; Pot *et al.*, 1994).

Table 1-4. Genera comprising the group of gram-positive organisms designated as lactic acid bacteria [a]

Carnobacterium	*Pediococcus* [b]
Enterococcus	*Streptococcus*
Lactobacillus	*Tetragenococcus* [b,d]
Lactococcus	*Vagococcus*
Leuconostoc	*Weissella* [e]
Oenococcus [c]	

[a] Adapted from Axelsson, 1993; Schleifer and Ludwig, 1995 and Vandamme *et al.*, 1996.

[b] Cell division in two planes, tetrad formation.

[c] Formerly *Leuconostoc oenos* (Dicks *et al.*, 1995).

[d] Formerly *Pediococcus halophilus*, extremely salt tolerant.

[e] Formerly *Leuconostoc paramesenteroides* and related species including lactobacilli.

Several LAB strains are aerotolerant and mechanisms responsible for this resistance to O_2 have been reported, including NADH oxidase activity which appears to be universally distributed among LAB (Condon, 1987). Non-heme-catalase termed "pseudocatalase" has been reported in *Lactobacillus, Pediococcus, Leuconostoc and Enterococcus,* and contributes to oxygen resistance (Engesser and Hammes, 1994). Furthermore, true heme-catalase activity has been reported to provide a source of heme (e.g., hematin/hemoglobin) is included in the growth medium because LAB are unable to produce porphyrin groups and they are thus described as catalase negative (Condon, 1987; Axelsson, 1993; Engesser and Hammes, 1994).

Tolerance of acidic conditions varies widely from one genus of LAB to another. *Lactobacillus* are the most acid tolerant, whereas the non-aciduric *Carnobacterium piscicola* cannot grow below pH 5.0 (Ahn and Stiles, 1990b; Pot *et al.,* 1994). LAB are typically nonsporeformers; however, sporeforming organisms like *Sporolactobacillus* are closely related to the currently defined LAB (Fritze and Claus, 1995). Motility is an unusual characteristic among LAB. Only *Vagococcus* spp. and *Carnobacterium mobile, Carnobacterium funditum* and *Carnobacterium alterfunditum* are motile.

Humans have consumed fermented foods for generations without apparent ill effects, hence it is assumed that LAB are not toxic. However, production of carcinogenic and mutagenic compounds during food fermentation has been observed (Nout, 1994) and LAB strains have been implicated in human infections (Aguirre and Collins, 1993). A study performed on mice indicated that *Lactobacillus casei* strain GG, *Streptococcus thermophilus* and *Lactobacillus helveticus* have no harmful or toxic effect even

at the highest dose fed i.e., 6 g of freeze-dried culture/kg. Bacteria were administrated by gavage and the animals remained under observation for 7 days. Punctual graduated doses of 1, 2, 4 or 6 g of bacteria/kg of body weight were tested. The equivalent for a 70 kg male is 450 g of bacteria. Daily consumption of 20 g of cheese and 400 g of yogurt is equivalent to about 1 to 2 g of bacteria, which is a few hundred times smaller than the equivalent amount calculated (Donohue *et al.,* 1993).

According to these results LAB appear to be non-toxic. In fact, health benefit claims have been made for consumption of food fermented with lactic acid bacteria. The first work on this subject was by the Nobel prize winner Elie Metchnikoff who observed that the Bulgarians had a higher proportion of centenarians in their population and that their diet was particularly rich in fermented milk. With Bélonowsky, he studied the "detoxifying" substances produced by the "Bulgar bacilli" (Metchnikoff, 1908).

Lactic acid bacteria, and more commonly *Lactobacillus* and *Enterococcus,* are part of the gastrointestinal microflora (Nousiainen and Setälä, 1993). The gastrointestinal microflora plays an important role in the development of the human immune system as demonstrated with axenic animals (Crabbe *et al.,* 1968; Spencer *et al.,* 1993). Several health benefits of LAB have been postulated (Nousiainen and Setälä, 1993):

- Competitive exclusion of pathogens (antimicrobial substances, adhesion to mucosae),
- Anticholesterolemic effect,
- Inhibition of carcinogenic fecal enzymes (β-glucuronidase,

nitroreductase, azoreductase),

- Deconjugation of bile,
- Growth performance and digestion efficiency,
- Stimulation of the immune system.

Unfortunately, LAB do not only have favorable attributes associated with them. Their involvement in endocarditis and other forms of bacteremia have tarnished their nonpathogenic status. Where LAB are involved in human infections, the patients were severely immunocompromised by surgical procedures or otherwise. Several incriminated isolates of *Leuconostoc* and *Enterococcus* were resistant to antibiotics including vancomycin.

The genus *Streptococcus* is very heterogeneous and includes highly pathogenic organisms, intestinal bacteria and the lactic starter cultures for cheese making but, based on molecular techniques, this genus has been divided into the following genera: *Streptococcus* sensu stricto, *Enterococcus* and *Lactococcus*. Classification of *S. thermophilus* with the *Lactococcus* could not be justified and it is the only food starter culture still included in this genus (Pot *et al.*, 1994). *Enterococcus* spp. have been associated as primary pathogens in various forms of human infections; whereas for the other LAB, it is not clear if they were secondary invaders or simply contaminants (Aguirre and Collins, 1993). The use of *Enterococcus faecalis* and *Enterococcus faecium* as probiotics is therefore questionable. It is contradictory that the same group of organisms can serve as probiotics and at the same time be potential pathogens. Is it simply a manifestation of the fragility of our ecological relationship with this group of organisms? Hopefully, future research will lead to a better understanding of this heterogeneous group of organisms.

1.4.1. The genus *Carnobacterium*

Because this thesis involves the study of bacteriocin production and regulation in *Carnobacterium piscicola* LV17, a brief description of this newly defined genus is presented. *Carnobacterium* is a member of the LAB that has been isolated from meat and meat products, poultry, fish, seawater and French surface-ripened soft-cheese (Millière *et al.*, 1994; Schillinger and Holzapfel, 1995). However, its natural habitat has not been firmly identified (Dainty and Mackey, 1992). The phylogenetic tree presented by Axelsson (1993) and Pot *et al.* (1994) positioned *Carnobacterium* as a member of the "*Enterococcus* group" which includes *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Tetragenococcus* and *Vagococcus*.

Species of the genus *Carnobacterium* have undergone extensive taxonomic change (Table 1-5). They were originally designated as *Streptobacterium* according to the classification proposed by Orla-Jensen (1919). *Carnobacterium* are described by Collins *et al.* (1987) as heterofermenters; however, their gas production is variable, limited and dependent on the growth medium. L-(+)-lactic acid is produced from glucose fermentation (Holzapfel and Gerber, 1983; Shaw and Harding, 1985). Glucose is metabolized by the glycolytic pathway and acetate, formate and CO_2 appear to be end-products of secondary degradation of pyruvate (De Bruyn *et al.*, 1987, 1988; Schillinger and Holzapfel, 1995). More work needs to be done to clarify the metabolism of this non-aciduric LAB.

Until recently, *Carnobacterium* spp. were designated as "atypical lactobacilli" because they share several characteristics with the lactobacilli.

Table 1-5. Retrospective of the taxonomy of *C. piscicola*

Designation	Reference
Streptobacterium	Orla-Jensen (1919)
"Atypical" lactobacilli	Thornley and Sharpe, 1959 Hitchener *et al.*, 1982 Shaw and Harding, 1984
Lactobacillus divergens [a]	Holzapfel and Gerber, 1983
Lactobacillus piscicola [b]	Hiu *et al.*, 1984
Lactobacillus carnis [b]	Shaw and Harding, 1985
Carnobacterium piscicola [c]	Collins *et al.*, 1987

[a] *Lb. divergens* included both *C. piscicola and C. divergens.*

[b] Collins *et al.* (1987) demonstrated that they were the same species.

[c] Collins *et al.* (1991) demonstrated that it has 100% sequence homology with 1340 nucleotides from the 16S rRNA generated from eubacterial specific primers (see text p. 23).

The first strains were isolated from irradiated poultry that was packaged anaerobically. They were discriminated from the typical lactobacilli by their poor growth on MRS medium and acetate agar that are commonly used as selective media for *Lactobacillus* (Thornley, 1957; Thornley and Sharpe, 1959; Collins *et al.*, 1987). Carnobacteria were also isolated from vacuum packaged beef, pork and lamb (Hitchener *et al.*, 1982; Shaw and Harding, 1984), and it soon became evident that these "atypical" lactobacilli were associated with vacuum-packaged meat (Shaw and Harding, 1985; Dainty and Mackey, 1992; Schillinger and Holzapfel, 1995).

In 1983, Holzapfel and Gerber isolated 120 psychrotrophic bacteria from vacuum packaged minced meat, of which 15 were described as lactobacilli; however, eight of these 15 lactobacilli were atypical heterofermenters and they were proposed as a new species, *Lactobacillus divergens.* Their proposal was based on the production of mainly L-(+)-lactic acid from hexoses and pentoses, their low mol% G + C (34 ± 0.8) and the presence of *meso*-diaminopimelic acid in the peptidoglycan. Shaw and Harding (1985) demonstrated by DNA hybridization that two closely related species could be identified. These two species also differ in their sugar fermentation patterns and their composition in cellular fatty acids. The *L. divergens* designation was maintained (now *Carnobacterium divergens*) and they named the second group *Lactobacillus carnis* (now *C. piscicola*). In the meantime, Hiu *et al.* (1984) isolated 17 bacterial strains from diseased fish which were somewhat related to the lactobacilli but could not be associated with any known species. They proposed the new species name *L. piscicola* for these fish isolates. Collins *et al.* (1987) demonstrated that *L. carnis* and *L. piscicola* were homologous and the *"piscicola"* designation prevailed. Subsequently, it was shown that *L.*

maltaromicus and *L. piscicola* have 100% sequence homology with 1340 nucleotides from the 16S rRNA generated from eubacterial specific primers (Collins *et al.*, 1991). Further analysis remains to be done before concluding that these taxa constitute a single species (Collins *et al.*, 1991; Schillinger and Holzapfel, 1995; Schleifer and Ludwig, 1995).

These "atypical" *Lactobacillus* were finally granted genus status as *Carnobacterium* based on their inability to grow on acetate agar and at low pH, the production of L-(+)-lactic acid as the predominant isomeric product of glucose fermentation and differences in cellular fatty acid and peptidoglycan composition (Collins *et al.*, 1987). Today, six species have been described: *C. divergens*, which is the type strain of *Carnobacterium*, *C. piscicola*, *C. mobile*, *C. gallinarum*, *C. funditum* and *C. alterfunditum* (Schillinger and Holzapfel, 1995). *C. piscicola* LV17 used in this study was originally isolated from vacuum packaged pork by Shaw and Harding (1984).

1.5. Bacteriocins of Lactic Acid Bacteria

1.5.1. Definition, classification and toxicity

From the first observation of antagonistic activity among bacteria by Pasteur and Joubert in 1877, to the discovery of penicillin by Alexender Fleming in 1929, microbiologists have devoted decades of research to finding new antimicrobial compounds produced by microorganisms. An early definition of the term "bacteriocin" was based on the nature of colicins produced by *E. coli* and pyocin produce by *Streptococcus pyogenes* compared with antibiotic substances (Jacob *et al.*, 1953). Characteristics of the colicins include:

- proteinaceous in nature
- lethal biosynthesis
- intraspecies killing activity (narrow spectrum of activity)
- adsorption to specific receptors on the surface of targeted/sensitive cells

As the knowledge of colicins and the bacteriocins produced by gram-positive bacteria expanded, the need for a new definition arose. Tagg *et al.* (1976) proposed a new definition based on the following six criteria:

- narrow spectrum of activity,
- presence of a biologically active protein moiety,
- bactericidal mode of action,
- binding to a specific receptor,
- plasmid mediated activity,
- lethal biosynthesis.

So many exceptions have been found to this definition that Konsky (1982) suggested that only two criteria should be used to describe a bacteriocin: the proteinaceous nature of the compound and the presence of an immunity mechanism towards the bacteriocin that is produced.

The search for "natural" antimicrobials for use in food systems as biopreservatives directed the research on bacteriocins towards LAB that are widely used in the food industry. The wealth of information gathered over two decades has lead to the grouping of bacteriocins into four distinct classes as described by Klaenhammer (1993):

I. Lantibiotics: small peptides containing unusual amino acids and lanthionine and β-methyllanthionine cyclic structures, such as nisin. Upon dehydration of serine or threonine residues, a thioether bond is formed with cysteine thereby forming the characteristic cyclic structure of this class of bacteriocins.

II. Small hydrophobic and heat-stable peptides with no unusual amino acids: the precursors of these bacteriocins are characterized by a Gly^{-2}-Gly^{-1}**$^{+1}$Xaa cleavage site. This class has been subgrouped as follows:

IIa. The *Listeria*-active peptides containing the consensus sequence -Tyr-Gly-Asn-Gly-Val-Xaa-Cys- (YGNGV motif) at the N-terminus of the molecule.

IIb. Complexes which necessitate two peptides for activity, e.g., lactacin F and lactococcin G (Nes *et al.*, 1996).

IIc. Thiol-activated peptides for which cysteine residues must be in a reduced state for activity.

III. Large heat labile proteins.

IV. Proteins that require another chemically different moiety (lipid, carbohydrate) for activity.

The lantibiotics can be subdivided into two groups according to the tertiary structure of the peptide (Bierbaum and Sahl, 1993):

Type A. Screw-shaped molecules with a net positive charge. Their inhibitory activity is due to their capacity to form pores in the cytoplasmic membrane of sensitive cells.

Type B. Globular molecules with no net charge or a net negative charge. They are enzyme inhibitors.

More recently, new bacteriocins with a signal peptide, instead of the typical N-terminal leader sequence with a Gly^{-2}-Gly^{-1}**+1Xaa cleavage site, were identified (Leer *et al.*, 1995; Worobo *et al.*, 1995; Tomita *et al.*, 1996). This new type of bacteriocin does not fit any of the subgroups of Class II bacteriocins described above although the mature peptide has the characteristics of a class II bacteriocin.

The vast majority of the bacteriocin-producing strains of LAB have been isolated from food (Schillinger *et al.*, 1993) and many LAB used in food fermentations produce bacteriocins (Ray, 1993). It can therefore be assumed that humans have been exposed to bacteriocins through their diet. This argument is not sufficient to demonstrate the innocuous nature of bacteriocins, especially if they are used as additives in concentrations higher than that naturally found in food. Because of their proteinaceous nature it is expected that they will be degraded by gastrointestinal enzymes (Schillinger *et al.*, 1993) and such enzymes are actually used to verify their nature.

A British group from the University of Birmingham investigated the toxicity of nisin on rats or guinea-pigs (Frazer *et al.*, 1962). Nisin was fed in the form of cheese produced from a nisin-producing starter culture (cheese was the

sole source of protein in the diet at levels of 20, 30 or 40%). A commercial preparation (Meganisin or a preparation of Meganisin hydrolysate with 0.1 N HCl) was mixed with the regular rodent feed. The authors calculated that if all foods other than bread in the diet of a 70 kg male are treated with nisin at a level of 20 units/g, the daily intake would be 140 units/kg. They converted that value for a rat of 250 g and referred to it as the D value. They either gave the rats 14 times or 1400 times the calculated D value. The following parameters were used to assess nisin toxicity:

- acute toxicity: tested by gastric incubation and intraperitoneal injection,
- body and organ weights
- reproductive performance and survival of the young,
- mortality and autopsy (up to 2 years),
- gastrointestinal, kidney and hepatic function,
- hematological investigations,
- sensitization of the ileum.

The results indicated that for the group of rats fed 14 times the D value of nisin, the growth rate was lower but this was attributed to reduced food intake because the 1400 times the D value group was normal. In generation 1, it was noticed that the females but not the males fed with the highest dose of nisin, had significantly larger kidneys, ovaries and uteri; however, no renal damage was observed even at the highest dose of nisin. Sensitization of the ileum only occurred when nisin was injected intraperitoneally, suggesting that nisin is degraded during gastrointestinal transit. All of the other parameters tested indicated that nisin is nontoxic even with the acute toxicity test. The mortality

test indicated that rats died in most cases of lung infections or infections of other types independent of the diet. No mention was made of the evaluation of nisin concentration in the cheese. The high rate of cannibalism reported (40%) indicates that the animals were not visited regularly during the study which could have influenced the results on mortality.

A second study done by a Japanese group using rats and mice indicated that no difference in growth, blood tests, weight, volume and histology of various organs was observed between the test and the control groups. In this study the animals were force-fed various concentrations of nisin for 3 months (Hara *et al.*, 1962). The toxicity of nisin was estimated to be similar to salt i.e., LD_{50} of 7 g/kg (Hurst, 1981).

The capacity of bacteriocins to elicit an humoral response (antibody production) was tested in various mammals. When the Class IIa bacteriocin pediocin AcH/PA1 produced by *Pediococcus acidilactici* H was partially purified by ammonium sulfate precipitation and was injected intraperitoneally with an adjuvant or as a bovine serum albumin conjugate, no antibodies were detected with either of the immunization methods used (Bhunia *et al.*, 1990). Tagg *et al.* (1976) attributed the weak antigenicity of bacteriocins to their low molecular weight. However, all polypeptides of > 1 kDa are immunogenic though at different levels (Bach, 1986). Pediocin AcH/PA1 has a molecular weight of 6.2 kDa (Ray and Hoover, 1993).

Polypeptide antibiotics like polymyxin can cause nephrotoxic effects and the presence of unusual amino acids in the structure of nisin has raised some concern regarding its toxicity (Frazer *et al.*, 1962). The early investigations of

Hara *et al.* (1962) and Frazer *et al.* (1962) indicated that nisin is a suitable food additive and furthermore, it was granted GRAS status (Generally Recognized As Safe) in the United States in 1988 (Fields, 1996). Like the carnobacteriocins produced by *C. piscicola* LV17, pediocin AcH/PA1 is a member of the class II bacteriocins. By analogy, the lack of antigenic properties for pediocin AcH/PA1 and the toxicological data obtained for nisin suggest that a similar low toxicity should be expected for carnobacteriocins A, B2 and BM1 (described later in Section 1.5.3. p. 36).

Despite the limited number of published papers on bacteriocin toxicity, nisin has been used as a food preservative in over 40 countries for products that vary from milk, to baby food, to canned vegetables (Delves-Broughton, 1990). Toxicological analyses are expensive and require a large quantity of the pure compound. These inconveniences might cause delays in commercial utilization of other bacteriocins if Regulatory Agencies require such analyses to be done before their acceptance as food additives (Gould, 1996).

1.5.2. Biochemical and genetic characteristics of bacteriocins produced by lactic acid bacteria: an overview.

Bacteriocin production can either be plasmid or chromosomally mediated. In the case of nisin, the whole operon (Fig. 1-1) is encoded on a large conjugative transposon containing the insertion element (IS*904*) immediately upstream of the nisin structural gene (Dodd and Gasson, 1994). The producer strains are protected from the inhibitory effect of the bacteriocin that they produce by a specific immunity protein, and its structural gene is usually located downstream of the bacteriocin gene (Dodd and Gasson, 1994).

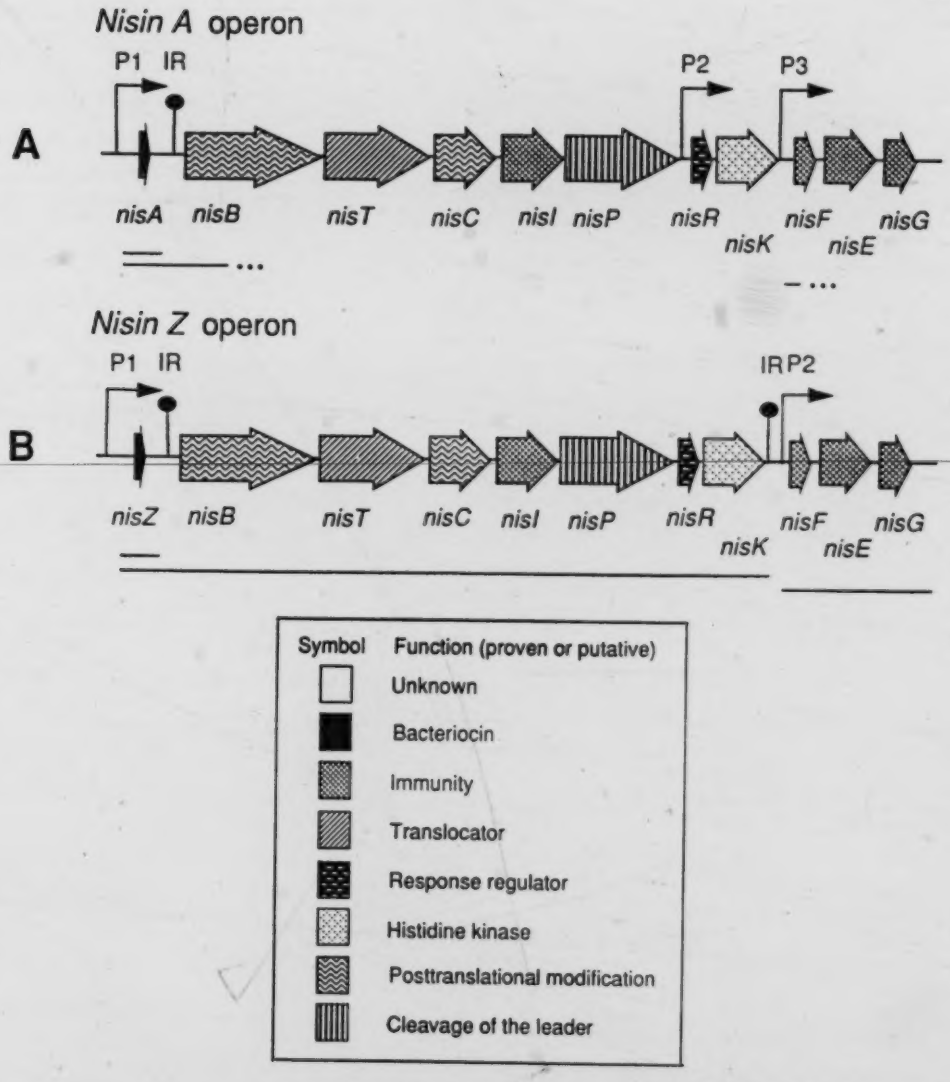


Fig. 1-1. Genetic map (14-kb) of the nisin A operon (A) according to Kuipers *et al.* (1995) and De Ruyter *et al.* (1996) and nisin Z operon (B) according to Ra *et al.* (1996) and Qiao *et al.* (1996). The genes of importance to this review are described in the text, Section 1.5.2 (p. 29). Transcripts are designated by solid lines below the operon map. Transcripts followed by ··· indicates that they were identified using promoter probe analysis and that the actual size of the transcript is not known. Promoter (┌➔), transcription terminator (❡), inverted repeat (IR).

Apart from their relatively small size (51 to 150 amino acids), the immunity proteins identified thus far share a limited amount of homology (Nes *et al.*, 1996). The mode of action of the immunity protein is not fully elucidated but it was proposed to act by shielding the receptor protein making it unrecognizable by the bacteriocin (Dodd and Gasson, 1994). With regard to nisin, more than one gene is apparently involved in the immunity process. The *nisI* gene is implicated in self-protection of the producer strain and the last three genes of the nis operon named *nisF, E* and *G* were found to complement immunity to nisin in an unexplained manner (Kuipers *et al.*, 1993; Siegers and Entian, 1995; Fig. 1-1). Sequence analysis of NisI indicated that it could be a membrane-bound lipoprotein located outside of the cell membrane and Engelke *et al.* (1994) postulated that the immunity protein (NisI) gives resistance by "floating" in the membrane to interact directly with the nisin molecules or to interfere with their polymerization during pore formation. The functions of *nisF, E* and *G* in the immunity process remain to be elucidated.

The ribosomally synthesized bacteriocins are translated as prepeptides with an N-terminal extension which is involved in secretion. The vast majority of the bacteriocins of LAB studied so far are secreted by a dedicated secretory machinery (sec-independent secretion pathway; Dodd and Gasson, 1994). With the exception of nisin, which only has one gene ascribed to secretion (Fig.1-1), secretory machinery is generally composed of two proteins which are essential for expression of the bacteriocin-positive phenotype. One of the proteins has homology with ATP-binding cassette (ABC) transporter proteins. Typically, these transporter proteins have two characteristic domains, a hydrophobic membrane-associated domain and a cytoplasmic ATP-binding domain and they work as homodimers. The second protein acts as an

accessory protein. Similar secretion systems have been identified in gram-negative organisms, e.g., hemolysins and colicin V (Marugg *et al.*, 1992; Stoddard *et al.*, 1992; Håvarstein *et al.*, 1995). However, a few bacteriocins have been found that have a typical signal peptide and they are thus secreted by the sec-dependent secretion pathway. Divergicin A produced by *C. divergens* (Worobo *et al.*, 1995), acidocin B produced by *Lactobacillus acidophilus* (Leer *et al.*, 1995) and bacteriocin 31 produced by *E. faecalis* which is encoded on a pheromone-responsive conjugative plasmid (Tomita *et al.*, 1996).

The cleavage of the N-terminal extension of the class II bacteriocins is performed by the ABC translocator protein which has a proteolytic domain in the cytoplasmic N-terminal part of the molecule (Håvarstein *et al.*, 1995). For nisin a specific protease, NisP, is responsible for the cleavage of the N-terminal part of the molecule after the precursor is translocated outside of the cell. When part of the *nisP* gene is deleted the precursor accumulates extracellularly. *E. coli* cell extracts overexpressing NisP are also able to cleave the precursor to its active mature form. Sequence analysis of NisP indicates the presence of a N-terminal secretory signal sequence, a catalytic domain and a putative C-terminal anchor suggesting that after translocation and maturation to an active form, the protein remains anchored in the cell membrane (Van der Meer *et al.*, 1993)

The first information available on bacteriocin regulation was based on the effect of growth conditions and their influence on bacteriocin production; a section of this thesis is devoted to this subject (1.6.1, p. 46). At the molecular level, genes encoding proteins with homology to components of the histidine

kinase class of sensing and signaling pathways have been identified. Autogenous regulation mechanisms were observed in a few producer strains and this is the subject of section 1.6.5 of this thesis (p. 57).

The bacteriocins of LAB studied thus far have a similar mode of action and their target is the cytoplasmic membrane. They act by increasing the permeability of the cell membrane by forming pores. These pores create an efflux of essential compounds and dissipate the proton motive force which leads to growth inhibition and eventually cell death. Some bacteriocins are not active on liposomes indicating that a receptor protein is needed as in the case of lactococcins A and B; whereas nisin is active against liposomes and therefore it does not appear to need a receptor protein. Pore formation by the bacteriocin can be voltage dependent as in the case of nisin or voltage independent as reported for lactococcins (Dodd and Gasson, 1994).

Nisin is active against vegetative sensitive cells by the pore formation mechanism described above. It is also active against bacterial spores by preventing their outgrowth, notably *Clostridium* spores. *Clostridium* spores are of particular concern in low acid thermally processed foods and in the bloating of cheese. Binding of the dehydroalanine at position 5 in the nisin molecule to sulfhydryl groups of membrane proteins prevents outgrowth of the spores (Dodd and Gasson, 1994). These reactive sulfhydryl groups are not available in ungerminated spores but they are present on freshly germinated spores (Morris *et al.*, 1984).

Many of the inhibitory substances listed in Table 1-1 are produced by LAB and they allow the LAB to compete and inhibit various spoilage and

pathogenic organisms including gram-negative bacteria. However, bacteriocins produced by LAB are generally effective against organisms closely related to the producer strains, and do not include gram-negative organisms because the outer membrane protects the cytoplasmic membrane from the cytotoxic effect of the bacteriocin. This protective effect is due to the lipopolysaccharide molecules that act as a barrier to hydrophobic compounds (Ray, 1993). Processes that alter the integrity of the outer membrane could be used to enhance bacteriocin efficiency. Cells of gram-negative bacteria that have been sublethally injured by heating, freezing or acid treatment became sensitive to the action of nisin and pediocin AcH/PA1. Efficiency to reduce the viability of the cells varied according to the challenged organism and the sublethal stress imposed (Kalchayanand *et al.*, 1992).

It is well established that calcium and magnesium ions are important for the stability of the outer membrane (reviewed by Ray, 1993). The effect of chelating agents on bacteriocin activity against gram-negative bacteria has been studied by two independent groups. One group studied the role of various chelating agents in making nisin inhibitory to *Salmonella* (Stevens *et al.*, 1991, 1992b; Shefet *et al.*, 1995). The efficiency of the treatment varied with the chelator used and the target organism, but of the chelators tested, EDTA was the most effective in killing of the gram-negative cells by nisin. Addition of bovine serum albumin was used to evaluate the influence of proteins on the chelator ability to improve the activity of nisin on gram-negative cells. No significant reduction of the inhibitory action of nisin used in combination with chelator was observed. No reduction of treatment efficiency was observed for the temperature range tested (4 to 42^{o}C), temperatures ≥ 30 ^{o}C were found to be the most effective. Using a combination of EDTA, citric

acid, Tween 20 (to study the influence of surfactant on nisin inhibitory properties) and nisin at pH 3.5, the shelf life of refrigerated broiler drumsticks was extended by 1.5 to 3 days compared with the control dipped in water for 30 min. Furthermore, the nisin-containing formulation were more effective in drumstick skin decontamination than 20 ppm of chlorine (USDA-accepted concentration for chiller thank; Shefet, *et al.,* 1995). The antimicrobial activity of nisin against *Salmonella typhimurium* lipopolysaccharide mutants confirmed that the integrity of the outer membrane is important for insensitivity in gram-negative bacteria to nisin (Stevens *et al.,* 1992a).

Another group obtained similar results (Cutter and Siragusa, 1995a). They were able to reduce the population of *E. coli* and *S. typhimurium* by 2.29 to 5.49 log cfu/ml depending on the treatment and the organism tested. Release of cellular components was evaluated by absorbance, but electron microscopy indicated that the treatment had a limited effect on the membrane. When similar treatments were applied on lean beef tissue, it was 10 times less efficient than *in vitro*, indicating that the meat constituents interfere with the action of either the chelator or the bacteriocin itself (Cutter and Siragusa, 1995b). Comparison of these studies indicates that the bacteriocin and chelator treatment is more effective on chicken skin than on lean beef tissue. However, use of Tween 20 on the chicken skin, might have increased the efficiency of the treatment due to its surfactant action on the cells.

There have been reports of bacteriocin produced by LAB being active against gram-negative bacteria. But failure to obtain a pure compound left reasonable doubt that other inhibitory compounds might be responsible for the effect. However, the situation with *Enterococcus faecium* ssp. *liquefaciens*

S-48 is different because the bacteriocin has been purified to homogeneity (Gálvez *et al.*, 1989a, b). Unfortunately the organism was not isolated from food but from human wound exudate. The bacteriocin-encoding gene was cloned and sequenced from a 68-kb plasmid (Martínez-Bueno *et al.*, 1990, 1994). It is a basic protein with a molecular weight of 7.2 kDa and it acts by permeabilization of the cytoplasmic membrane as observed for other LAB bacteriocins (Gálvez *et al.*, 1991). The pure bacteriocin could not be analyzed by Edman degradation most probably due to its N-terminus being blocked. The oligonucleotide probes used to localize the bacteriocin structural gene were synthesized based on the Edman degradation results from peptides obtained by hydrolysis of the pure compound with Glu-C endoproteinase. The amino acid sequence of the hydrolyzates revealed that the molecule is a head-tail cyclic compound and its nucleotide sequence indicates that the molecule is ribosomally synthesized from a prepeptide which loses its N-terminal extension during the maturation process. This N-terminal extension has a characteristic DNA sequence of signal peptides (Martínez-Bueno *et al.*, 1994). Lanthionine and unusual amino acids were not detected indicating that the bacteriocin is not a lantibiotic (Gálvez *et al.*, 1989b). The head-tail cyclic structure is an unusual feature and the authors speculate that it might be responsible for the effect against gram-negative bacteria. This is the first report of a LAB bacteriocin that is active against gram-negative organisms without simultaneous treatment to injure the outer membrane of the target organism.

1.5.3. Bacteriocins produced by *Carnobacterium* spp. and more particularly by *C. piscicola* LV17

Schillinger and Holzapfel (1990) were the first to report bacteriocin

production in *Carnobacterium* spp. Of the 37 strains tested, 18 produced bacteriocins that had a narrow spectrum of activity, primarily against other *Carnobacterium* spp. Since then, more food isolates have been tested, several bacteriocins have been identified and some have been analyzed at the molecular level (Table 1-6).

C. piscicola LV17 was isolated from vacuum packaged pork. It produces three bacteriocins designated carnobacteriocins A, B2 and BM1 (Ahn and Stiles, 1990a, b and 1992; Quadri *et al.*, 1994; Worobo *et al.*, 1994). They are interesting bacteriocins because they are active against strains of *Listeria* and *Enterococcus* (Ahn and Stiles, 1990a) and bacteriocin production is detected early in the growth cycle of a 1% inoculum in synthetic medium (Ahn and Stiles, 1990b). The structural genes of the three bacteriocins have been cloned and sequenced (Quadri *et al.*, 1994; Worobo *et al.*, 1994) and other genes essential for bacteriocin production have been identified (Quadri, 1996; Worobo, 1996; Fig. 1-2).

1.5.3.1. Carnobacteriocin A

Carnobacteriocin A is ribosomally synthesized as a 71 amino acid prepeptide that is modified by cleavage of an 18 amino acid leader sequence at a typical double glycine cleavage site (class II bacteriocin; Fig. 1-3). Cleavage of the N-terminal extension releases a hydrophobic peptide of 5052.85 Da. The carnobacteriocin A operon is located on a 72-kb plasmid, pCP49, and a 9.6-kb *Pst*I fragment was found to be essential for bacteriocin production and immunity (Worobo, 1996). A genetic map of the 7 open reading frames sequenced from the 9.6-kb *Pst* fragment is presented in Fig. 1-2A. The

Table 1-6. Bacteriocins produced by *Carnobacterium* spp.

Strains	Isolated from	Bacteriocin	Bacteriocin class [a]	Gene location	References
UI49	fish	carnocin UI49	I	unknown	Stoffels *et al.*, 1992
LK5	raw ground beef	not named	unknown	unknown	Buchanan and Klawitter, 1992
LV17	VP [b] pork	carnobacteriocin A	II	72-kb plasmid	Worobo *et al.*, 1994
		B2	IIa	62-kb plasmid	Quadri *et al.*, 1994
		BM1	IIa	chromosome and 62-kb plasmid	Quadri *et al.*, 1994
LV61	VP lamb	piscicolin 61 [c]	unknown	22-kb plasmid	Schilinger *et al.*, 1993
JG126	spoiled ham	piscicolin 126	IIa	unknown	Jack *et al.*, 1996
CP5	foodstuffs	carnocin CP5	unknown	chromosome	Mathieu *et al.*, 1993
VI	fish products	piscicocin VI	unknown	unknown	Pilet *et al.*, 1995
C. divergens					
V41	fish products	divercin V41	unknown	unknown	Pilet *et al.*, 1995
LV13	VP pork	divergicin A	II	3.4-kb plasmid	Worobo *et al.*, 1995

[a] Bacteriocin class as defined in the text p. 25.

[b] Vacuum packaged.

[c] Piscicolin 61 is identical to carnobacteriocin A (Holck *et al.*, 1994).

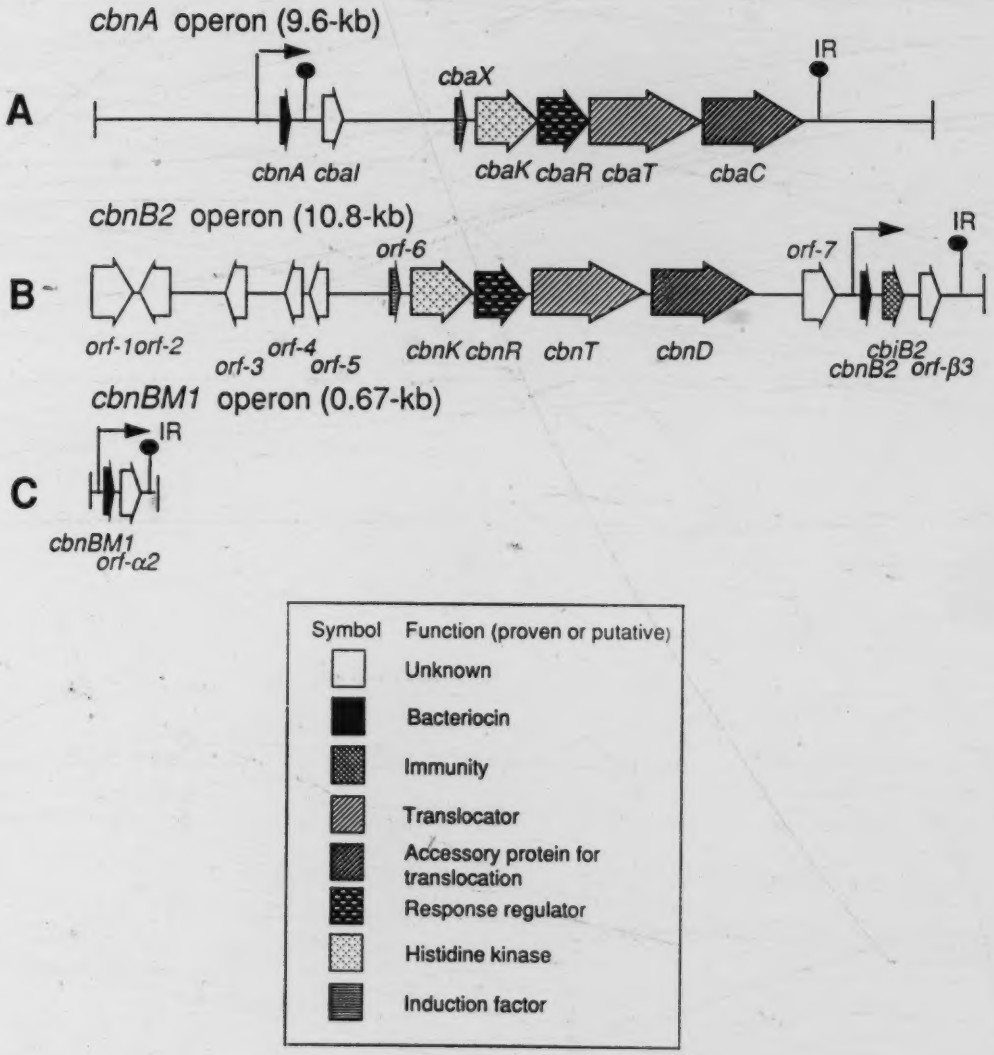


Fig. 1-2. Genetic map of carnobacteriocin A (A), B2 (B) and BM1 (C) according to Quadri (1996) and Worobo (1996). The genes of importance to this review are described in the text, Section 1.5.3 (p. 36). Promoter (↱), transcription terminator (↟), inverted repeat (IR).

CbnA [a]	MNNVKELSIKEMQQVTGG/DQMSDGVNYGKGSSLSKGGAKCGLGIVGGLATIPSGPLGWLAGAAGVINSCMK
CbnB2 [b]	MNSVKELNVKEMKQLHGG/VNYGNGVSCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP
CbnBM1 [b]	MKSVKELNKKEMQQINGG/AISYGNGVYCNKEKCWVNKAENKQAITGIVIGGWASSLAGMGH

Fig. 1.3. Amino acid sequence of CbnA, CbnB2 and CbnBM1. ⬇ indicated the leader sequence cleavage site. Underlined methionines are succeptible to oxidation (Quadri *et al.*, 1994; Worobo et al., 1994). [a] Worobo, 1996; [b] Quadri *et al.*, 1994.

genes involved in immunity have not been fully characterized. The immunity protein is usually located immediately downstream of the bacteriocin structural gene (Dodd and Gasson, 1994). But when *cbaI* was cloned by itself in front of the constitutive promoter, P32, it did not provide immunity to the transformed cells. However, immunity was achieved by cloning the 3.2-kb *Eco*RI-*Xba*I fragment which contains *cbaX*, *cbaK*, *cbaR* and the N-terminus of *cbaT*, suggesting that more than one gene may be involved in immunity (Worobo, 1996), as in the case of nisin.

CbaX has homology to *orf-4* of the sakacin A operon and *orf-6* of *cbnB2* operon. The next two genes encode proteins that have homology to the histidine kinase class of sensing and signaling pathways (CbaK and CbaR), whereas CbaT and CbaC have homology to ATP-dependent transport proteins. Conserved cysteine and histidine residues were shown to be present in the leader sequence proteolytic cleavage site of the ABC translocator protein of lactococcin G (Håvarstein *et al.*, 1995) and such conserved residues are also present in CbaT (Worobo, 1996).

1.5.3.2. Carnobacteriocin B2

Similar to carnobacteriocin A, carnobacteriocin B2 is a class II bacteriocin but it contains the conserved YGNGV motif which defines the bacteriocins of subclass a. The bacteriocin contains 48 amino acids and has a molecular mass of 4969.9 Da (Quadri *et al.*, 1994; Fig. 1-3). The *cbnB2* operon is located on a 62-kb plasmid, pCP40, and a 10-kb *Pst*I fragment contains the essential information for bacteriocin production and immunity (Fig. 1-2B; Quadri, 1996).

cbiB2 encodes the immunity protein for CbnB2. No other gene seems to be needed to protect the producer strains. Immunoblot analysis indicated that the immunity protein is mostly located intracellularly and that the protein does not bind to CbnB2 (Quadri *et al.*, 1995). The genes *cbnK, cbnR, cbnT* and *cbnD* have similar postulated functions as those described for *cbaK, cbaR, cbaT* and *cbaC* in the carnobacteriocin A operon (Quadri, 1996). The *orf-6* has homology to plantaricin A (Diep *et al.*, 1994) and IF (induction factor, *orf-Y*) in the sakacin P operon (Eijsink *et al.*, 1996) and it is currently under investigation to determine the functional similarities with plantaricin A and IF from the sakacin P operon (Quadri *et al.*, unpublished; Section 1.6.3, p. 51). The role of *orf-6* in bacteriocin production is discussed in further details in the general conclusion (p. 181). The other small genes present in the 10-kb *Pst*I-*Pst*I fragment have not been ascribed any function; however, *orf*-1 and *orf*-2 have homology to transposases and *orf*-4 and 5 have N-terminal extensions with a Gly-Gly motif similar to those of CbnB2 and CbnBM1 (Quadri, 1996).

1.5.3.3. Carnobacteriocin BM1

CbnBM1 is similar to CbnB2 and it also has the YGNGV motif. It contains 43 amino acids and has a molecular mass of 4524.6 (Fig. 1-3). Contrary to the other two bacteriocins, it is chromosomally encoded (Fig.1-2C). A putative immunity function has been proposed for the *orf*-α2 located downstream of the bacteriocin structural gene (Quadri *et al.*, 1994). However, homology of *orf*-α2 with *orf*-3 of the bacteriocin 31 operon indicates that it probably has other functions because in the bacteriocin 31 system, the immunity gene was found to be *bacB*, the gene upstream of *orf*-3 (Tomita *et al.*, 1996). Although the structural gene is located on the chromosome,

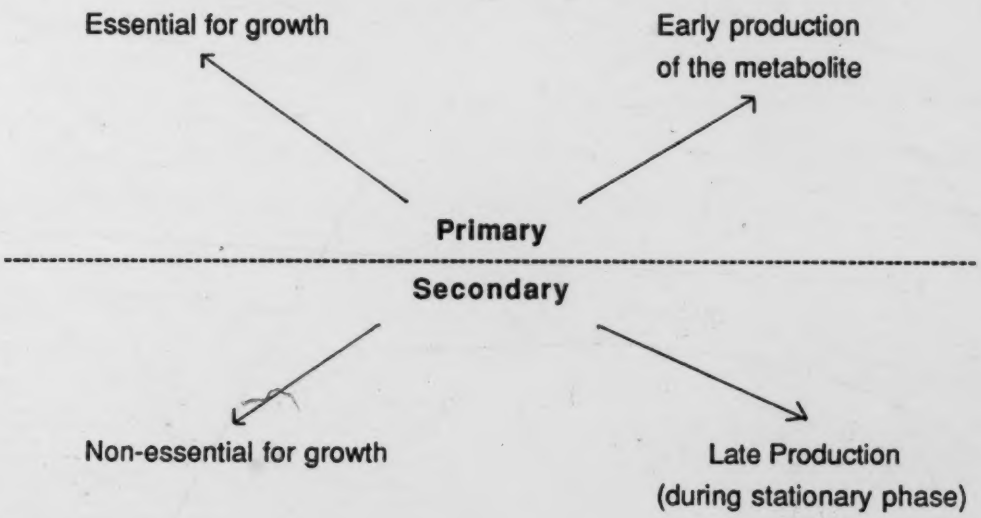
carnobacteriocin BM1 production is dependent on the presence of pCP40 (Quadri, 1996; Chapter 3)

1.5.4. Are bacteriocins secondary or primary metabolites?

In a recent paper, De Vuyst and coworkers (1996) postulated that bacteriocin production by *Lactobacillus amylovorus* follows "primary metabolite kinetics" based on the following observations:

- a peak of activity is observed during the mid-exponential phase,
- bacteriocin production is coupled to growth rate: bacteriocin production is enhanced at slow growth rate due to unfavorable conditions.

Definitions of primary and secondary metabolites vary according to the references used, most probably due to the terms themselves:



Mann (1978) defined primary metabolites as substances "essential for the survival and well-being" of the organism that produces them. Secondary metabolites are defined as a "natural products with no apparent utility for the producer organism." Assessing the "utility" of a secondary metabolite for the producing organism outside of its natural habitat can be difficult. Production of an antimicrobial compound could have some "utility" in competing for the nutrients within a specific ecological niche (Klaenhammer, 1988; Dykes, 1995). Based on this utility criterion, there is no such thing as a secondary metabolite.

Mann (1987) added to his definition of secondary metabolites that "they are perhaps only activated during a particular stage of growth and development, or during periods of stress caused by nutritional limitation or microbial attack." According to this, amylovorin L471 described by De Vuyst *et al.* (1996) is not a primary metabolite. The definition given by Maplestone *et al.* (1992) also reflects the idea that secondary metabolites are non-essential for growth: "substance which does not play an explicit role in the internal economy of the organism that produces it."

The definition of secondary metabolites given by Davies (1992) is mostly based on biological function associated with secondary metabolites. According to this definition, bacteriocins would be included because they act as "competitive weapons against other bacteria, etc. (self-protection/exclusion)." The biological functions associated with secondary metabolites are said to be related to the "interaction between the organism and its environment... concerned with what is going on outside the producing organism" as opposed to primary metabolites that are linked to "events going on inside" the cell.

Definition of primary and secondary metabolites based on their production according to growth phase has also been used (Tortora *et al.*, 1992). Primary metabolite production coincides with active growth phase (trophophase) whereas secondary metabolites are not produced until growth is almost complete (idiophase), but this definition has been challenged because of conflicting examples.

Vining (1992) conveys the flexibility of the definitions presented thus far:

> "From a broad perspective, secondary metabolic products might be expected to benefit the producing organism in either of two ways: they might improve its ability to grow, reproduce or disperse in appropriate conditions, or they might afford protection from competition or predation. The majority of compounds fit into these categories."

Carnobacteriocins are detected early in the growth cycle of *C. piscicola* LV17 (Ahn and Stiles, 1990b) and their production only occurs if they are induced before the stationary phase of growth (Chapter 2). They could be considered primary metabolites according to the trophophase/idiophase definition of Tortora *et al.* (1992). However, the plasmidless variant that does not produce bacteriocin grows just as well as the wild-type strain under laboratory conditions. Carnobacteriocin production is not essential for survival of the organism and would therefore qualify as a secondary metabolite. In its natural habitat, bacteriocin production in *C. piscicola* LV17 might be essential for survival. Because evolution and synthesis of secondary metabolites are influenced by the natural habitat of the producer, a proper definition of primary

and secondary metabolites might not be achieved without specifying the growth conditions under which a metabolite is studied (Cavalier-Smith, 1992; Maplestone *et al.*, 1992; Vining, 1992).

1.6. Regulation of bacteriocin production

1.6.1. Growth conditions and bacteriocin production

Growth conditions have been shown to influence bacteriocin production. Bacteriocin is produced within a specific pH range which varies with producer strains and can be quite different from the pH range at which the bacteriocin is stable and active (De Vuyst and Vandamme, 1992; Kaiser and Montville, 1993; Mørtvedt-Abildgaard *et al.*, 1995). *C. piscicola* LV17 grows at pH > 5.0 but bacteriocin is produced only at pH > 5.6, and carnobacteriocins A, B2 and BM1 are active at a pH range of 1 to 9 (Ahn and Stiles, 1990a; Quadri *et al.*, 1994; Worobo *et al.*, 1994). Controlled pH fermentation usually gives better bacteriocin production (De Vuyst and Vandamme, 1992; Kaiser and Montville, 1993).

Buchanan and Klawitter (1992) observed a temperature dependent effect on inhibition of *L. monocytogenes* Scott A by *C. piscicola* LK5. They attributed the inhibition not only to the reduced growth rate of the target strain but also to the production of a heat stable bacteriocin. However, they did not test for bacteriocin production at low temperature. De Vuyst *et al.* (1996) observed an improved specific production of amylovorin L471 when the temperature was low and unfavorable for growth, and suggested that bacteriocin production in *L. amylovorus* is conditioned by temperature stress.

Nisin is produced by *Lactococcus lactis* ssp. *lactis* NIZO 22186 during exponential growth, and it was postulated that carbon source in the medium regulates its production (De Vuyst and Vandamme, 1992). Nutritional stress due to iron and glucose limitation improved production of colicin V produced by *E. coli* and bavaricin produced by *Lactobacillus "bavaricus"* MN, respectively (Chehade and Braun, 1988; Kaiser and Montville, 1993). Certain compounds were found to improve bacteriocin production when present in the growth medium (Table 1-7). In our laboratory, a difference in bacteriocin production was observed when APT medium from two different suppliers was tested (BBL, Becton-Dickinson Microbiology Systems, Cockeysville, MD and Difco Laboratories, Detroit, MI). Change in the source of yeast extract in the commercial product (BBL) improved bacteriocin production from a low inoculum. This is in contradiction to Schillinger *et al.* (1993) who did not observe an effect of yeast extract on bacteriocin production. The improved production of bacteriocin in the presence of Tween 80 (Huot *et al.*, 1996) might involve the reduction of bacteriocin adherence to the producer cell which, in the case of nisin, is associated with bacteriocin synthesis (Meghrous *et al.*, 1992). Growth conditions for optimum bacteriocin production vary from one producer organism to another (Parente and Hill, 1992). Bacteriocin production was also achieved in continuous culture (Meghrous *et al.*, 1992) including with calcium alginate-immobilized bacteria (Wan *et al.*, 1995).

1.6.2. Bacteriocinogenicity in liquid vs. solid medium

Early work to find bacteriocin-producing strains of lactic acid bacteria showed that the method used to identify producer strains had a great influence on the percentage of producers identified among the strains tested. When

Table 1-7. Growth medium components which promote bacteriocin production

Compound	Bacteriocin and producer strains	Reference
amino acids	Nisin/*Lactococcus lactis*	Kozak and Dobrzanski, 1977
beef extract	Bavaricin MN/ *Lactobacillus "bavaricus"* MN	Kaiser and Montville, 1993
Tween 80	Bacteriocin J46/*Lactococcus lactis* subsp. *cremoris* J46	Huot *et al.*, 1996
peptone	Piscicolin 61 [a]/*C. piscicola* LV61	Schillinger *et al.*, 1993

[a] Piscicolin 61 is identical to carnobacteriocin A (Holck *et al.*, 1994).

studying strains of *Lactobacillus fermenti* for bacteriocin production, De Klerk (1967) observed that more strains were positive when tested on solid medium compared with using the cell-free supernatant to inhibit a target strain. Geis *et al.* (1983) confirmed these observations when they demonstrated that 23% of 280 strains of lactic streptococci that they tested inhibited growth of other streptococci using the agar test compared with 16% using the cell-free supernatant.

Some bacteriocinogenic strains only produce bacteriocin when grown on solid media, for example lactacin B produced by *L. acidophilus* (Barefoot and Klaenhammer, 1983), plantacin B produced by *L. plantarum* NCDO 1193 (West and Warner, 1988) and propionicin PLG-1 produced by *Propionibacterium thoenii* (Lyon and Glatz, 1991). Production of staphylococcin 1580 by *Staphylococcus epidermidis* was detected in liquid medium but the quantity produced was so small that purification was not practical (Jetten *et al.*, 1972). In fact, staphylococcin 1580 was purified from a semi-solid medium containing 0.4 % agar where the activity was 20 times higher than that in liquid medium.

Rammelsberg and Radler (1990) also reported that the spectrum of bacteriocin activity in *Lactobacillus brevis* B 26 varied when tested with growth on solid vs. liquid medium. Six out of nine target organisms were inhibited when the bacteriocin activity was tested on solid medium compared with one out of nine with cell-free supernatant. Bacteriocins diffuse more readily in liquid than on solid medium. In the latter, bacteriocins are concentrated and reach the threshold concentration necessary to inhibit less sensitive target strains.

The relevance of this variation of bacteriocin production on solid vs. liquid media for the producer strain is not known. However, many examples of bacterial behavior in Nature associated with growth on solid surfaces has been described. For instance, there are three conditions that must occur before cells of myxobacteria produce fruiting bodies and spore-bearing apparatus: the cells must undergo partial starvation; they must be on a solid surface; and they must be present at high cell density. A set of 5 signals referred to as A, B, C, D, and E are involved in social behavior and development, including formation of fruiting bodies. Contact-mediated interactions are important for the transduction of C and E signals and the cells must be in contact for these cellular signals to take place whereas in the slime mold *Dictyostelium*, cell contact is not necessary. Phosphate and adenosine secreted in the surrounding medium have also been investigated as developmental signaling molecules (Dworkin, 1991b, 1996). Another interesting aspect of the multicellularity of myxobacteria is the cooperative feeding or "wolf-pack effect" of growth on casein. Growth rate on casein is dependent on cell density, whereas growth rate on low molecular weight compounds is independent of cell density. The life cycle of myxobacteria is based on a "crowded type of life style" which could be an early evolutionary development towards multicellularity (Dworkin, 1991b).

Importance of growth on solid medium for bacteriocin production in *C. piscicola* LV17 is part of the investigation included in this thesis and it is discussed in subsequent chapters.

1.6.3. Bacteriocin production as a function of cell density

Despite their unicellular nature, bacteria often function, interact and communicate as a "community" for achievement of a common goal (Dworkin, 1991a). Some bacteria have a mechanism that allows them to monitor and evaluate their own population, referred to as "quorum sensing." This form of intercellular communication coordinates specific activity at a specific cell density. This phenomenon has been observed in several bacterial genera and for the following functions (Kaiser and Losick, 1993; Fuqua *et al.*, 1994; Salmond *et al.*, 1995):

- colonization of light organs by *Phosphobacterium fischeri* (formerly, *Vibrio fischeri*);
- morphological differentiation and antibiotic production in *Streptomyces griseus*;
- conjugal transfer of Ti plasmids in *Agrobacterium tumefaciens*;
- extracellular virulence factor from *Pseudomonas aeruginosa;*
- production of cell-wall-degrading enzymes in *Erwinia carotovora;* and
- regulation of rhizosphere genes in *Rhizobium leguminosarum.*

These systems have homoserine lactone-type autoinducers in common which diffuse freely outside of the cell and serve as the signaling molecule that monitors cell density. This particular system allows *P. ficheri* to differentiate when it is living at low cell density (< 10^2 cell/ml) in sea water compared with its symbiotic association at high cell density (10^9 to 10^{10} cells/ml) with the light organ of certain fish and squid (Dunlup and Greenberg, 1991). At the molecular level, a transcriptional activator, LuxR, has been identified and

requires the homoserine lactone autoinducer for its regulatory activity. Although no direct biochemical evidence has been provided, it is believed that the transcriptional activator needs to bind to the cell density signal molecule to activate transcription of the bioluminescence genes (Salmond *et al.*, 1995). Bioluminescence is not only regulated by the autoinducer but also by other factors associated with a slower growth rate, such as low concentration of O_2, low concentration of iron and cAMP (glucose limitation). Such conditions are believed to be part of the internal environment of the light emitting organs (Dunlup and Greenberg, 1991). In myxobacteria, the quorum sensing indicators are amino acids and short peptides referred to as the A signal (Dworkin, 1996; Kaiser, 1996)

Among the lactic acid bacteria, conjugation in *Enterococcus* species occurs through a pheromone-induced clumping process which brings the donor and recipient cells into close proximity. The pheromone secreted by the recipient is a low-molecular-weight hydrophobic peptide of generally less than 10 amino acids, specific to the conjugative plasmid to be transferred. This pheromone induces production of cell surface adhesins on the donor cell which bind to the chromosomally-encoded binding substance, probably the lipoteichoic acid of the recipient cell thus forming a large aggregate of recipient and donor cells in close contact that facilitates plasmid transfer (Dunny, 1991).

In *C. piscicola* LV17, bacteriocin production occurs in liquid medium only if the culture is grown from a high cell density inoculum of $\geq 10^6$ cfu/ml. In a fully grown culture obtained from a nonbacteriocin-producing inoculum ($\leq 10^4$ cfu/ml), bacteriocin-positive phenotype is restored if the cells are grown on solid media (Saucier *et al.*, 1995). Subsequently, dependence of bacteriocin

production on growth from a high cell density inoculum was also observed in *L. plantarum* C11 and in *L. sake* (Diep *et al.*, 1995; Eijsink *et al.*, 1996).

L. plantarum C-11 was isolated from cucumber fermentation. The antagonistic compound in the supernatant of the producer strain is heat stable (>30 min at 100^{o}C) and it is active against four genera of lactic acid bacteria: *Lactobacillus, Pediococcus, Leuconostoc* and *Streptococcus* (Daeschel *et al.*, 1990). The bacteriocin, designated plantaricin A (PlnA), was tentatively purified using ammonium sulfate precipitation followed by cationic exchange, separation by Octyl-Sepharose and finally by reverse-phase HPLC chromatography (Nissen-Meyer *et al.*, 1993). However, what was originally thought to be the bacteriocin is in fact an inducer of bacteriocin production in a nonbacteriocin-producing low inoculum and is still designated as PlnA (Diep *et al.*, 1995). It is referred to as a bacteriocin-like compound because it has a leader sequence similar to class II bacteriocins. The structural gene responsible for the antimicrobial activity has not been identified yet, however several other genes have homology to class II bacteriocins and they are indicated in Fig. 1-4.

Northern analysis revealed that *plnA* is co-transcribed with 3 down-stream genes referred to as *plnB, C* and *D* (Fig. 1-4) which show homology with genes of the accessory gene regulatory (*agr*) system in *Staphylococcus aureus*. PlnB, PlnC and PlnD have homology to components of the histidine kinase class of sensing and signaling pathways. PlnB is homologous to the histidine kinase whereas PlnC and PlnD have homology to the response regulator. The *agr* system is responsible for the growth phase-dependent control and expression of exoproteins and some of these proteins are involved

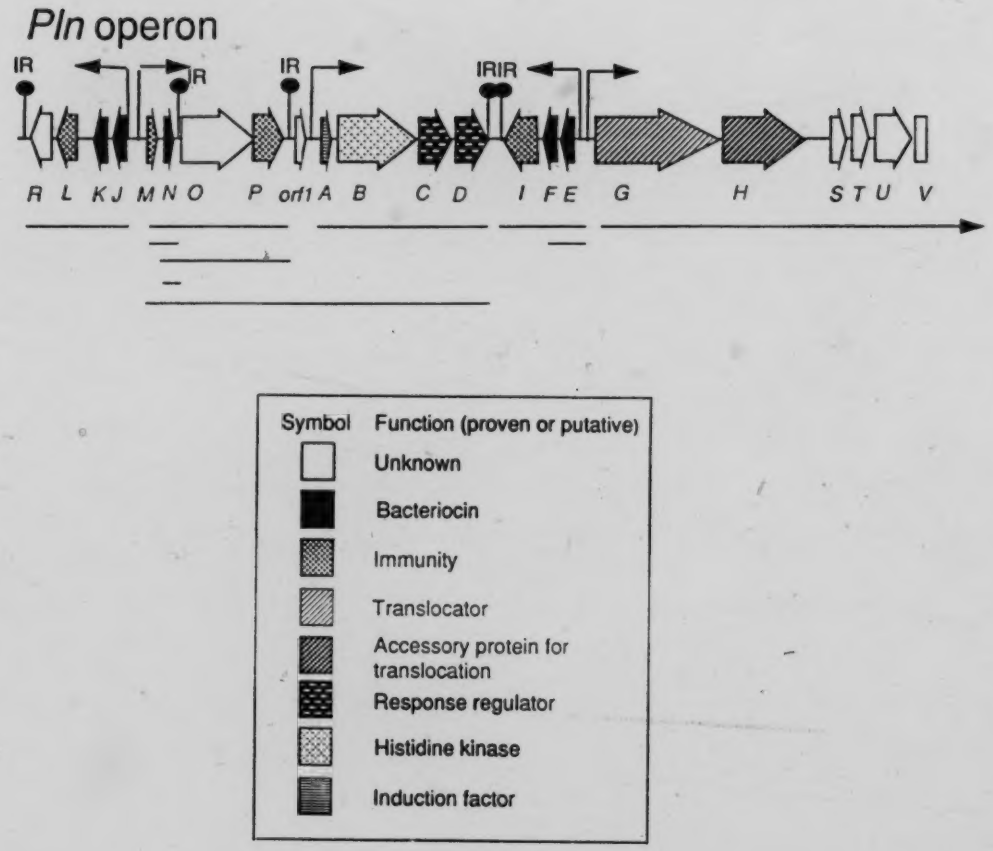


Fig. 1-4. Genetic map of the operon for bacteriocin production in *L. plantarum* C11 (16.1-kb) according to Diep *et al.* (1996). The genes of importance to this review are described in the text, Section 1.6.3 (p. 51). Transcripts induced by PlnA are indicated by solid lines. An arrow at the end of a solid line indicates that the transcript continues beyond the known sequence. Promoter (⌐►), transcription terminator (Ŧ), inverted repeat (IR).

in the pathogenicity of the organism (Diep *et al.*, 1994). Plantaricin A acts as the bacteriocin inducer in low inoculum culture. It activates its own transcription, which is on the same transcriptional unit as *plnBCD,* and other adjacent regulons (Fig. 1-4, Diep *et al.*, 1996).

Similar results were also found in *L. sake* LTH673 that produces sakacin P (Eijsink *et al.*, 1996). A major difference exists between the two *Lactobacillus* systems and the *Carnobacterium* system: bacteriocin production is recovered readily by growth on solid medium in the case of *Carnobacterium* whereas for *L. plantarum* C11 and *L. sake*, the inducers, plantaricin A and IF, have to be added to the solid medium for recovery of the bacteriocin-positive phenotype. This observation suggests a different regulation mechanism between these two systems.

1.6.4. Role of bacteriocin in regulatory function.

The first report of a regulatory function for bacteriocins produced by LAB was presented by Hurst (1981) for the lantibiotic nisin produced by *Lactococcus lactis* ssp. *lactis*. Based on results obtained by addition of nisin to growing producer cells at different stages in the growth cycle, he postulated that nisin is involved in regulating "the beginning and the end of growth" in batch culture. He also reported that strains that do not produce nisin synthesize other peptides, similar to nisin, which would have a similar function in the cell. By inactivation of the respective structural genes, Kuipers *et al.* (1993) and Reis *et al.* (1994) demonstrated that nisin and Pep5 production are essential for their immune phenotype. These results led Bierbaum *et al.* (1994) to postulate that the lantibiotic prepeptide could be involved in both regulatory

and autoregulatory processes. The mechanism of this regulatory function of nisin on the immunity protein is apparently due to a transcriptional read through of the immunity protein from the *nisA* promoter, which is autoregulated (Kuipers *et al.*, 1995; see below).

In addition to pore formation, nisin inhibits murein synthesis by formation of a complex with the lipid intermediate I (Reisinger *et al.*, 1980), and both nisin and Pep 5 induce autolysis in a sensitive target organism (Bierbaum and Sahl, 1985, 1987). Autolytic activity is controlled by lipoteichoic acids and stimulation of enzyme activity by small quantities of Pep5 or nisin at low ionic strength was observed if teichoic or teichuronic acids are present in the cell walls.

These reports indicate a regulatory role, apart from the antimicrobial activity, for bacteriocins produced by LAB. Antimicrobial compounds that have a regulatory function are not uncommon. Pamamycin-607 produced by *Streptomyces alboniger* IFO 12738 possesses antimicrobial activity against some phytopathogenic fungi and gram-positive bacteria, and it also induces production of aerial mycelium in the producer. A minimum concentration of 0.1 mg/disk is necessary for induction of mycelium formation and induction increases with increasing concentration up to a limit of 10 mg/disk at which point it starts inhibiting formation of mycelium. Pamamycin-607 is part of a complex of eight homologues for which the MW varies from 593 to 691. The variations in biological activity of the homologues (aerial mycelium induction, substrate mycelium inhibition) indicate a structure-function relationship which remains to be elucidated (Kondo *et al.*, 1988).

1.6.5. Autoregulation of bacteriocin production.

Bacteriocin autoregulation was first reported in *C. piscicola* LV17 using purified bacteriocins to induce bacteriocin production in a low inoculum nonproducing bacteriocin ($\leq 10^4$ cfu/ml; Chapter 2). Subsequently, the necessity of nisin A production for expression of other genes in the *nisA* operon was confirmed by gene disruption and induction with pure nisin (Kuipers, *et al.*, 1995; De Ruyter *et al.*, 1996). A 4-bp deletion in the *nisA* structural gene abolished transcription of that gene unless a subinhibitory quantity of nisin was added to the growing culture. Mature nisin obtained by proteolytic processing of 23 amino acids from the N-terminus of the precursor can induce transcription. The active domain for induction is in the N-terminal part of the molecule as demonstrated by use of mutants and fragments of the mature peptide (Kuipers *et al.*, 1995). Ra *et al.* (1996) and Qiao *et al.* (1996) obtained similar results with nisin Z and confirmed the autoregulatory function of nisin. The results also demonstrated that the amount of immunity protein and the level of immunity is proportional to the nisin concentration used for induction (Ra *et al.*, 1996).

The promoter P2 in front of *nisR* (Fig. 1-1A) and the promoter P3 in front of *nisF* (Fig. 1-1A) were tested for nisin-induced activity; NisR has homology with the response regulator of the histidine kinase class of sensing and signaling pathways and NisF is involved in the immunity of the producer cell towards nisin. The *nisR* promoter is independent of the presence of nisin and has a constitutive behavior whereas the promoter in front of *nisF* is dependent on the presence of nisin, similar to the promoter in front of *nisA* (P1, Fig. 1-1A; Kuipers *et al.*, 1995; De Ruyter *et al.*, 1996). Similarities in the promoter

sequence of *nisA* and *nisF* have been reported (De Ruyter *et al.*, 1996).

In the case of nisin Z produced by *Lactococcus lactis* N8, there are two promoters, one in front of *nisZ* (Fig. 1-1B; P1) and the other in front of *nisF* (Fig. 1-1B; P2) that were found to be nisin dependent. The particular genetic organization of the nisin operon in *L. lactis* N8 (Ra *et al.*, 1996) differs from the system presented by Kuipers *et al.* (1995) for nisin A because a promoter was not found in front of *nisR*.

The *nisK* gene was found to be essential for *nisA* transcription and its disruption abolished the inducibility of the *nisA* gene by nisin (Kuipers *et al.*, 1995). NisR and NisK have homology to proteins of the histidine kinase class of sensing and signaling pathways which are ubiquitous in bacteria (Falke *et al.*, 1995). It was postulated that nisin exerts its autoregulation by activating NisK which phosphorylates itself, then transfers the phosphoryl group to NisR which acts as a transcriptional activator of the promoter in front of *nisA* and *nisF* (Kuipers *et al.*, 1995). Proteins that have homology with components of the histidine kinase class of sensing and signaling pathways have also been identified in CbnA and CbnB2 operons (Quadri, 1996; Worobo, 1996).

1.7. Research objectives

Studies on the concentrations of cells of *C. piscicola* LV17 necessary to inhibit the growth of a target organism *C. divergens* LV13 indicated that bacteriocin production in *C. piscicola* LV17 is dependent on inoculation level and that bacteriocin production from a low inoculum can be recovered by growth on a solid medium (Saucier *et al.*, 1995). The general objective of this

study was to understand how cell-dependent bacteriocin production occurs at the physiological and molecular level, and how it affects the potential for use of *C. piscicola* LV17 as a protective culture in meat systems.

The first objective was to determine the factors that would restore bacteriocin production from low inocula in liquid medium. The results detailed in Chapter 2 indicate that the carnobacteriocins autoregulate their own production provided that induction occurs prior to the stationary phase of growth.

The second objective was to determine at the molecular level how bacteriocin production at low inocula was controlled. It was found that bacteriocin production was controlled at the transcription level and the promoters responsible for bacteriocin production were identified. This is detailed in Chapter 3.

In order to use a microorganism as a bacteriocinogenic protective culture on meat packaged in modified atmosphere, low inoculation level must be applied otherwise the meat would spoil faster compared to the length of time that the original indigenous lactic microflora would have taken to spoil it. Can *C. piscicola* LV17 produce bacteriocin on meat at low inocula, in the same manner that it does on solid agar medium? The results detailed in Chapter 4 indicate that bacteriocin production at low inocula is possible on meat particles and pork fat at low inocula and that *C. piscicola* LV17 has the potential for use as a bacteriocinogenic protective culture in meat systems.

1.8. References

Ahn, C. and Stiles, M.E. 1990a. Antibacterial activity of lactic acid bacteria isolated from vacuum-packaged meats. Journal of Applied Bacteriology 69:302-310.

Ahn, C. and Stiles, M.E. 1990b. Plasmid-associated bacteriocin production by a strain of *Carnobacterium piscicola* from meat. Applied and Environmental Microbiology 56:2503-2510.

Ahn, C. and Stiles, M.E. 1992. Mobilization and expression of bacteriocin plasmids from *Carnobacterium piscicola* isolated from meat. Journal of Applied Bacteriology 73:217-228.

Aguirre, M. and Collins, M.D. 1993. Lactic acid bacteria and human clinical infection. Journal of Applied Bacteriology 75:95-107.

Axelsson, L.T. 1993. Lactic acid bacteria: classification and physiology. In: Salminen, S. and von Wright, A. (Ed.), Lactic acid bacteria. Marcel Dekker, Inc., New York, NY, pp. 1-63.

Bach, J.-F. 1986. Bases immunochimiques de l'antigénicité. In: Bach, J.-F., (Ed.), Immunologie, Flammarion Médecine-Sciences, Paris, pp. 101-127.

Ballongue, J. 1993. Bifidobacteria and probiotic action. In: Salminen, S. and von Wright, A. (Ed.), Lactic acid bacteria. Marcel Dekker, Inc., New York, NY, pp. 357-428.

Barefoot, S.F. and Klaenhammer, T.R. 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Applied and Environmental Microbiology 45:1808-1815.

Bhunia, A.K., Johnson, M.C., Ray, B. and Belden, E.L. 1990. Antigenic property of pediocin AcH produced by *Pediococcus acidilactici* H. Journal of Applied Bacteriology 69:211-215.

Bierbaum, G. and Sahl, H.-G. 1985. Induction of autolysis of staphylococci by the basic peptide antibiotics Pep5 and nisin and their influence on the activity of autolytic enzymes. Archives in Microbiology 141:249-254.

Bierbaum, G. and Sahl, H.-G. 1987. Autolytic system of *Staphylococcus simulans* 22: Influence of cationic peptides on activity of *N*-acetylmuramoyl-L-alanine amidase. Journal of Bacteriology 169:5452-5458.

Bierbaum, G. and Sahl, H.-G. 1993. Lantibiotics - Unusually modified bacteriocin-like peptides from gram-positive bacteria. Zentralblatt für Bakteriologie 278:1-22.

Bierbaum, G., Reis, M., Szekat, C. and Sahl, H.-G. 1994. Construction of an expression system for engineering of the lantibiotic Pep5. Applied and Environmental Microbiology 60:4332-4338.

Buchanan, R.L. and Klawitter, L.A. 1992. Characterization of a lactic acid bacterium, *Carnobacterium piscicola* LK5, with activity against *Listeria monocytogenes* at refrigeration temperatures. Journal of Food Safety 12:199-217.

Cavalier-Smith, T. 1992. Origins of secondary metabolism. In: Chadwick, D.J. and Whelan, J. (Ed.), Secondary metabolites: their function and evolution. Ciba Foundation Symposium 171. John Wiley and Sons, Chichester, England, pp. 64-87.

Chehade, H. and Braun, V. 1988. Iron-regulated synthesis and uptake of colicin V. FEMS Microbiology Letters 52:177-182.

Collins, M.D., Farrow, J.A.E., Phillips, B.A., Ferusu, S. and Jones, D. 1987. Classification of *Lactobacillus divergens*, *Lactobacillus piscicola*, and some catase-negative, asporogenous, rod-shaped bacteria from poultry in a new genus, *Carnobacterium*. International Journal of Systematic Bacteriology 37:310-316.

Collins, M.D., Rodrigues, U. Ash, C., Aguirre, M., Farrow, J.A.E., Martinez-Murcia, A., Phillips, B.A., Williams, A.M. and Wallbanks, S. 1991. Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. FEMS Microbiology Letters 77:5-12.

Condon, S. 1987. Responses of lactic acid bacteria to oxygen. FEMS Microbiology Reviews 46:269-280.

Crabbe, P.A., Bazin, H., Eyssen, H. and Heremans, J.F. 1968. The normal microbial flora as a major stimulus for proliferation of plasma cells synthesizing IgA in the gut. International Archives in Allergy 34:362-375.

Crawford, R.J.M. 1958. Cheese cultures, past, present and future. Journal of the Society of Dairy Technology 10:23-28.

Cutter, C.N. and Siragusa, G.R. 1995a. Population reductions of gram-negative pathogens following treatments with nisin and chelators under various conditions. Journal of Food Protection 58:977-983.

Cutter, C.N. and Siragusa, G.R. 1995b. Treatment with nisin and chelators to reduce *Salmonella* and *Escherichia coli* on beef. Journal of Food Protection 57:1028-1030.

Daeschel, M.A., McKenney, M.C. and McDonald, L.C. 1990. Bactericidal activity of *Lactobacillus plantarum* C-11. Food Microbiology 7:91-98.

Dainty, R.H. and Mackey, B.M. 1992. The relationship between the phenotypic properties of bacteria from chill-stored meat and spoilage processes. Journal of Applied Bacteriology 73:103S-114S.

Daniels, J.A., Krishnamurthi, R. and Rizvi, S.S.H. 1985. A review of effects of carbon dioxide on microbial growth and food quality. Journal of Food Protection 48:532-537.

Davies, J. 1992. Introduction. In: Chadwick, D.J. and Whelan, J. (Ed.), Secondary metabolites: their function and evolution. Ciba Foundation Symposium 171. John Wiley and Sons, Chichester, England, pp. 1-2.

De Bruyn, I.N., Louw, I.A., Visser, L. and Holzapfel, W.H. 1987. *Lactobacillus divergens* is a homofermentative organism. Systematic and Applied Microbiology 9:173-175.

De Bruyn, I.N., Holzapfel, W.H., Visser, L. and Louw, I.A. 1988. Glucose metabolism by *Lactobacillus divergens*. Journal of General Microbiology 134:2130-2109.

De Klerk, H.C. 1967. Bacteriocinogeny in *Lactobacillus fermenti*. Nature 214:609.

Delves-Broughton, J. 1990. Nisin and its uses as a food preservative. Food Technology 44(11):100, 102, 104, 106, 108, 111-112, 117.

De Ruyter, P.G.G.A., Kuipers, O.P., Beerthuyzen, M.M., Van Alen-Boerrigter, I. and de Vos, W.M. 1996. Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*. Journal of Bacteriology 178:3434-3439.

De Vuyst L. and Vandamme, E.J. 1992. Influence of the carbon source on nisin production in *Lactococcus lactis* subsp. *lactis* batch fermentation. Journal of General Microbiology 138:571-578.

De Vuyst, L., Callewaert, R. and Crabbé, K. 1996. Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. Microbiology (UK) 142:817-827.

Dicks, L.M.T., Dellaglio, F. and Collins, M.S. 1995. Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni* [corrig.] gen. nov., comb. nov. International Journal of Systematic Bacteriology 45:395-397.

Diep, D.B., Håvarstein, L.S. and Nes, I.F. 1995. A bacteriocin-like peptide induces bacteriocin synthesis in *Lactobacillus plantarum* C11. Molecular Microbiology 18:631-639.

Diep, D.B., Håvarstein, L.S. and Nes, I.F. 1996. Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* C11. Journal of Bacteriology 178:4472-4483.

Diep, D.B., Håvarstein, L.S., Nissen-Meyer, J. and Nes, I.F. 1994. The gene encoding plantaricin A, a bacteriocin from *Lactobacillus plantarum* C11, is located on the same transcription unit as an *agr*-like regulatory system. Applied and Environmental Microbiology 60:160-166.

Donohue, D.C., Deighton, M. and Ahokas, J.T. 1993. Toxicity of lactic acid bacteria. In: Salminen, S. and von Wright, A. (Ed.), Lactic acid bacteria. Marcel Dekker, Inc., New York, NY, pp. 307-313.

Dodd, H.M. and Gasson, M.J. 1994. Bacteriocins of lactic acid bacteria. In: Gasson, M.J. and de Vos, W.M. (Ed.), Genetics and biotechnology of lactic acid bacteria. Blackie Academic and Professional, London, pp. 211-251.

Dunlup, P.V. and Greenberg, E.P. 1991. Role of intercellular chemical communication in the *Vibrio fischeri* - monocentrid fish symbiosis. In: Dworkin, M. (Ed.), Microbial Cell-Cell Interactions. American Society for Microbiology, Washington, DC, pp. 220-253.

Dunny, G.M. 1991. Mating interactions in gram-positive bacteria. In: Dworkin, M. (Ed.), Microbial Cell-Cell Interactions. American Society for Microbiology, Washington, DC, pp. 9-33.

Dworkin, M. 1991a. Introduction. In: Dworkin, M. (Ed.), Microbial Cell-Cell Interactions. American Society for Microbiology, Washington, DC, pp. 1-6.

Dworkin, M. 1991b. Cell-cell interactions in myxobacteria. In: Dworkin, M. (Ed.), Microbial Cell-Cell Interactions. American Society for Microbiology, Washington, DC, pp. 179-216.

Dworkin, M. 1996. Recent advances in the social and developmental biology of the myxobacteria. Microbiological Reviews 60:70-102.

Dykes, G.A. 1995. Bacteriocins: ecological and evolutionary significance. Trends in Ecology and Evolution 10:186-189.

Eijsink, V.G.H., Brurberg, M.B., Middelhoven, P.H. and Nes, I.F. 1996. Induction of bacteriocin production in *Lactobacillus sake* by a secreted peptide. Journal of Bacteriology 178:2232-2237.

Engelke, G., Gutowski-Eckel, Z., Kiesau, P., Siegers, K., Hammelmann, M. and Entian, K.-D. 1994. Regulation of nisin biosynthesis and immunity in *Lactococcus lactis* 6F3. Applied and Environmental Microbiology 60:814-825.

Engesser, D.M. and Hammes, W.P. 1994. Non-heme catalase activity of lactic acid bacteria. Systematic and Applied Microbiology 17:11-19.

Falke, J.J., Blair, D.F., Silhavy, J.T. and Schmitt, R. 1995. BLAST 1995: International conference on bacterial locomotion and signal transduction. Molecular Microbiology 16:1037-1050.

Fang, T.J. and Lin, L.-W. 1994. Growth of *Listeria monocytogenes* and *Pseudomonas fragi* on cooked pork in a modified atmosphere packaging/nisin combination system. Journal of Food Protection 57:479-485.

Fields, F.O. 1996. Use of bacteriocins in food: regulatory considerations. Journal of Food Protection 59 suppl.:72-77.

Frazer, A.C., Sharratt, M. and Hickman, J.R. 1962. The biological effects of food additives. Journal of the Science of Food and Agriculture 13:32-42.

Fritze, D. and Claus, D. 1995. Spore-forming, lactic acid producing bacteria of the genera *Bacillus* and *Sporolactobacillus*. In: Wood, B.J.B. and Holzapfel, W.H. (Ed.). The genera of lactic acid bacteria. Blackie Academic and Professional, London, pp. 368-391.

Fuqua, W.C., Winans, S.C. and Greenberg, E.P. 1994. Quorum sensing in bacteria: LuxR-LuxI family of cell density response transcriptional regulators. Journal of Bacteriology 176:269-275.

Gálvez, A., Maqueda, M., Martínez-Bueno, M. and Valdivia, E. 1989a. Bactericidal and bacteriolytic action of peptide antibiotic AS-48 against gram-positive and gram-negative bacteria and other organisms. Research in Microbiology 140:57-68.

Gálvez, A., Giménez-Gallego, G., Maqueda, M. and Valdivia, E. 1989b. Purification and amino acid composition of peptide antibiotic AS-48 produced by *Streptococcus* (*Enterococcus*) *faecalis* subsp. *liquefaciens* S-48. Antimicrobial Agents and Chemotherapy 33:437-441.

Gálvez, A., Maqueda, M., Martínez-Bueno, M. and Valdivia, E. 1991. Permeation of bacterial cells, permeation of cytoplasmic and artificial membrane vesicles, and channel formation on lipid bilayers by peptide antibiotic AS-48. Journal of Bacteriology 173:886-892.

Geis, A., Singh, J. and Teuber, M. 1983. Potential of lactic streptococci to produce bacteriocin. Applied and Environmental Microbiology 45:205-211.

Gill, C.O. and Bryant, J. 1992. The contamination of pork with spoilage bacteria during commercial dressing, chilling and cutting of pig carcasses. International Journal of Food Microbiology 15:51-62.

Gill, C.O. and Newton, K.G. 1978. The ecology of bacterial spoilage of fresh meat at chill temperatures. Meat Science 2:207-217.

Gould, G.W. 1992. Ecosystem approaches to food preservation. Journal of Applied Bacteriology 73:58S-68S.

Gould, G.W. 1995. Biodeterioration of foods and an overview of preservation in the food and dairy industries. International Biodeterioration and Biodegradation 36:267-277.

Gould, G.W. 1996. Industry perspectives on the use of natural antimicrobials and inhibitors for food applications. Journal of Food Protection 59 suppl.:82-86.

Gravani, R.B. 1987. The cause and costs of foodborne disease. Dairy and Food Sanitarian 7:20-25.

Greer, G.G. 1989. Red meats, poultry, and fish. In: McKellar, R.C. (Ed.), Enzymes of psychrotrophs in raw food. CRC Press, Inc., Boca Raton, FL, pp. 267-292.

Hara, S., Yakazu, K., Nakakawaji, K., Takeuchi, T., Kobayashi, T., Sata, M., Imai, Z. and Shibuya, T. 1962. An investigation of toxicity of nisin with particular reference to experimental studies of its oral administration and influences on digestive enzymes. Journal of the Tokyo Medical College 20:175-207.

Håvarstein, L.S., Diep, D.S. and Nes, I.F. 1995. A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export. Molecular Microbiology 16:229-240.

Hitchener, B.J., Egan, A.F. and Rogers, P.J. 1982. Characteristics of lactic acid bacteria isolated from vacuum-packaged beef. Journal of Applied Bacteriology 52:31-37.

Hiu, S.F., Holt, R.A., Sriranganathan, N., Seidler, R.J. and Fryer, J.L. 1984. *Lactobacillus piscicola*, a new species from salmonid fish. International Journal of Systematic Bacteriology 34:393-400.

Holck, A.L., Axelsson, L. and Schillinger, U. 1994. Purification and cloning of piscicolin 61, a bacteriocin from *Carnobacterium piscicola* LV61. Current Microbiology 29:63-68.

Holzapfel, W.H. and Gerber, E.S. 1983. *Lactobacillus divergens* sp. nov., a new heterofermentative *Lactobacillus* species producing L(+)-lactate. Systematics in Applied Microbiology 4:522-534.

Huot, E., Barrena-Gonzalez, C. and Petitdemange, H. 1996. Tween 80 effect on bacteriocin synthesis by *Lactococcus lactis* subsp. *cremoris* J46. Letters in Applied Microbiology 22:307-310.

Hurst, A. 1981. Nisin. Advances in Applied Microbiology 27:85-123.

Jack, R.W., Wan, J., Gordon, J., Harmark, K., Davidson, B.E., Hillier, A.J., Wettenhall, E.H.R., Hickey, M.W. and Coventry, M.J. 1996. Characterization of the chemical and antimicrobial properties of piscicolin 126, a bacteriocin produced by *Carnobacterium piscicola* JG126. Applied and Environmental Microbiology 62:2897-2903.

Jacob, F. Lwoff, A., Siminovitch, A. and Wollman, E. 1953. Définition de quelques termes relatifs à la lysogénie. Annales de l'Institut Pasteur (Paris) 84:222-224.

Jay, J.M. 1992. Modern Food Microbiology, 4th ed. Chapman and Hall, New York, NY.

Jetten, A.M., Vogels, G.D. and De Windt, F. 1972. Production and purification of a *Staphylococcus epidermidis* bacteriocin. Journal of Bacteriology 112:235-242.

Kaiser, A.L. and Montville, T.J. 1993. The influence of pH and growth rate on production of the bacteriocin, bavaricin MN, in batch and continuous fermentations. Journal of Applied Bacteriology 75:536-540.

Kaiser, D. 1996. Bacteria also vote. Science 272:1598-1599.

Kaiser, D. and Losick, R. 1993. How and why bacteria talk to each other. Cell 73:873-885.

Kalchayanand, N., Ray, B., Field, R.A. and Johnson, M.C. 1989. Spoilage of vacuum-packaged refrigerated beef by *Clostridium*. Journal of Food Protection 52:424-426.

Kalchayanand, N., Hanlin, M.B. and Ray, B. 1992. Sublethal injury makes gram-negative and resistant gram-positive bacteria sensitive to the bacteriocins, pediocin AcH and nisin. Letters in Applied Microbiology 15:239-243.

Klaenhammer, T.R. 1988. Bacteriocins of lactic acid bacteria. Biochimie 70:337-349.

Klaenhammer, T.R. 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiology Reviews 12:39-86.

Kondo, S., Yasui, K., Natsume, M., Katayama, M. and Marumo, S. 1988. Isolation, physico-chemical properties and biological activity of pamamycin-607, an aerial mycelium-inducing substance from *Streptomyces alboniger*. The Journal of Antibiotics 9:1196-1204.

Konsky, J. 1982. Colicins and other bacteriocins with established modes of action. Annual Review in Microbiology 36:125-144.

Kozak, W. and Dobrzanski, W.T. 1977. Growth requirements and the effect of organic components of the synthetic medium on the biosynthesis of antibiotic nisin in *Streptococcus lactis* strain. Acta Microbiologica Polonica 26:361-368.

Kuipers, O.P., Beerthuyzen, M.M., De Ruyter, P.G.G.A., Luesink, E.J. and de Vos, W.M. 1995. Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. The Journal of Biological Chemistry 270:27299-27304.

Kuipers, O.P., Beerthuyzen, M.M., Siezen, R.J. and de Vos, W.M. 1993. Characterization of the nisin gene cluster *nisABTCIPR* of *Lactococcus lactis*: Requirement of expression of the *nisA* and *nisI* genes for development of immunity. European Journal of Biochemistry 216:281-291.

Lambert, A.D., Smith, J.P. and Dodds, K.L. 1991a. Combined effect of modified atmosphere packaging and low-dose irradiation on toxin production by *Clostridium botulinum* in fresh pork. Journal of Food Protection 54:94-101.

Lambert, A.D., Smith, J.P. and Dodds, K.L. 1991b. Effect of headspace CO_2 concentration on toxin production by *Clostridium botulinum* in MAP, irradiated fresh pork. Journal of Food Protection 54:588-592.

Lambert, A.D., Smith, J.P. and Dodds, K.L. 1991c. Effect of initial O_2 and CO_2 and low-dose irradiation on toxin production by *Clostridium botulinum* in MAP fresh Pork. Journal of Food Protection 54:939-944.

Lambert, A.D., Smith, J.P. and Dodds, K.L. 1991d. Shelf life extension and microbiological safety of fresh meat - a review. Food Microbiology 8:267-297.

Leer R.J., van der Vossen, J.M.B.M., van Giezen, M., van Noort, J.M. and Pouwels, P.H. 1995. Genetic analysis of acidocin B, a novel bacteriocin produced by *Lactobacillus acidophilus*. Microbiology (UK) 141:1629-1635.

Lyon, W.J. and Glatz, B.A. 1991. Partial purification and characterization of a bacteriocin produced by *Propionibacterium thoenii*. Applied and Environmental Microbiology 57:701-706.

Maga, J.A. 1988. Antimicrobial properties of smoke. In: Smoke in food processing. CRC Press Inc., Boca Raton, Fl., pp. 107-112.

Mann, J. 1978. Introduction. In: Mann, J. (Ed.), Secondary metabolism. Oxford University Press, London, pp.1-20.

Mann, J. 1987. Introduction. In: Mann, J. (Ed.), Secondary metabolism, 2nd edition. Oxford University Press, London, pp.1-24.

Maplestone, R.A., Stone, M.J. and Williams, D.H. 1992. The evolutionary role of secondary metabolites - a review. Gene 115:151-157.

Martínez-Bueno, M., Gálvez, A., Valdivia, E. and Maqueda M. 1990. A transferable plasmid associated with AS-48 production in *Enterococcus faecalis*. Journal of Bacteriology 172:2817-2818.

Martínez-Bueno, M., Maqueda M., Gálvez, A., Samyn, B., Van Beeumen, J., Coyette, J. and Valdivia, E. 1994. Determination of the gene sequence and the molecular structure of the enterococcal peptide antibiotic AS-48. Journal of Bacteriology 176:6334-6339.

Marugg, J.D., Gonzalez, C.F., Kunka, B.S., Ledeboer, A.M., Pucci, M.J., Toonen, M.Y., Walker, S.A., Zoetmulder, L.C.M. and Vandenbergh, P.A. 1992. Cloning, expression, and nucleotide sequence of genes involved in production of pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0. Applied and Environmental Microbiology 58:2360-2367.

Mathieu, F., Michel, M. and Lefebvre, G. 1993. Properties of a bacteriocin produced by *Carnobacterium piscicola* CP5. Biotechnology Letters 15:587-590.

McMullen, L.M. and Stiles, M.E. 1993. Microbiology of fresh pork stored under modified atmosphere at -1, 4 and 10^{o}C. International Journal of Food Microbiology 18:1-14.

Meghrous, J. Huot, E., Quittelier, M. and Petitdemange, H. 1992. Regulation of nisin biosynthesis by continuous cultures and by resting cells of *Lactococcus lactis* subsp. *lactis*. Research in Microbiology 143:879-890.

Metchnikoff, E. 1908. The prolongation of life. G.P. Putman's Sons, New York, NY.

Millière, J.B., Mathieu, M.M.F. and Lefebvre, G. 1994. Presence of *Carnobacterium* spp. in French surface mould-ripened soft-cheese. Journal of Applied Bacteriology 76:264-269.

Miller Jones, J. 1992. Food Safety. Eagan Press, St-Paul, MN.

Morris, S.L., Walsh, R.C. and Hansen, J.N. 1984. Identification and characterization of some bacterial membrane sulfhydryl groups which are targets of bacteriostatic and antibiotic action. Journal of Biological Chemistry 259:13590-13594.

Mørtvedt-Abildgaard, C.I., Nissen-Meyer, J., Jelle, B., Grenov, B., Skaugen, M. and Nes, I.F. 1995. Production and pH-dependent bactericidal activity of lactocin S, a lantibiotic from *Lactobacillus sake* L45. Applied and Environmental Microbiology 61:175-179.

Nes, I.F., Diep, D.B., Håvarstein, L.S., Brurberg, M.B., Eijsink, V. and Holo, H. 1996. Biosynthesis of bacteriocins in lactic acid bacteria. Antonie van Leeuwenhoek 70:113-128.

Nissen-Meyer, J., Larsen, A.G., Sletten, K., Daeschel, M. and Nes, I.F. 1993. Purification and characterization of plantaricin A, a *Lactobacillus plantarum* bacteriocin whose activity depends on the action of two peptides. Journal of General Microbiology 139:1973-1978.

Nousiainen, J. and Setälä J. 1993. Lactic acid bacteria as animal probiotics. In: Salminen, S. and von Wright, A. (Ed.), Lactic acid bacteria. Marcel Dekker, Inc., New York, NY, pp. 315-356.

Nout, M.J.R. 1994. Fermented foods and food safety. Food Research International 27:291-298.

Orla-Jensen, S. 1919. The lactic acid bacteria. Andr. Fred. Høst and Son, Copenhagen.

Parente, E. and Hill, C. 1992. A comparison of factors affecting the production of two bacteriocins from lactic acid bacteria. Journal of Applied Bacteriology 73:290-298.

Pilet, M.-F., Dousset, X., Barré, R., Novel, G., Desmazeaud, M. and Piard, J.-C. 1995. Evidence for two bacteriocins produced by *Carnobacterium piscicola* and *Carnobacterium divergens* isolated from fish and active against *Listeria monocytogenes*. Journal of Food Protection 58:256-262.

Pot, B., Ludwig, W., Kersters, K. and Schleifer, K.-H. 1994 Taxonomy of lactic acid bacteria. In: De Vuyst, L. and Vandamme, E.J. (Ed.), Bacteriocins of lactic acid bacteria. Chapman and Hall, London, pp. 13-90.

Qiao, M., Ye, S., Koponen, O., Ra, R., Usabiaga, M., Immonen, T. and Saris P.E.J. 1996. Regulation of the nisin operons in *Lactococcus lactis* N8. Journal of Applied Bacteriology 80:626-634.

Quadri, L.E.N. 1996. Biochemical and genetic characterization of antimicrobial peptides produced by *Carnobacterium piscicola* LV17B. Ph.D. thesis, University of Alberta, Edmonton, AB.

Quadri, L.E.N., Sailer, M., Roy, K.L., Vederas, J.C. and Stiles, M.E. 1994. Chemical and genetic characterization of bacteriocins produced by *Carnobacterium piscicola* LV17B. The Journal of Biological Chemistry 269:12204-12211.

Quadri, L.E.N., Sailer, M., Terebiznik, M.R., Roy, K.L., Vederas, J.C. and Stiles, M.E. 1995. Characterization of the protein conferring immunity to the antimicrobial peptide carnobacteriocin B2 and expression of carnobacteriocins B2 and BM1. Journal of Bacteriology 177:1144-1151.

Quadri, L.E.N., Kleerebezem, M., Kuipers, O.P., De Vos, W.M., Roy, K.L., Vederas, J.C. and Stiles, M.E. Genes from *Carnobacterium piscicola* LV17B involved in bacteriocin production and immunity: evidence for global transcriptional regulation and a novel inducer peptide. Unpublished.

Ra, S.R., Qiao, M., Immonen, T., Pujana, I. and Saris P.E.J. 1996. Genes responsible for nisin synthesis, regulation and immunity form a regulon of two operons and are induced by nisin in *Lactococcus lactis* N8. Microbiology (UK) 142:1281-1288.

Rammelsberg, M. and Radler, F. 1990. Antibacterial polypeptides of *Lactobacillus* species. Journal of Applied Bacteriology 69:177-184.

Rammelsberg, M., Müller, E. and Radler, F. 1990. Caseicin 80: purification and characterization of a new bacteriocin from *Lactobacillus casei*. Archives in Microbiology 154:249-252.

Ray, B. 1992. The need for biopreservation. In: Ray, B. and Daeschel, M. (Ed.) Food biopreservatives of microbiological origin. CRC Press, Inc., Boca Raton, FL, pp. 1-23.

Ray, B. 1993. Sublethal injury, bacteriocins, and food microbiology. ASM News 59:285-291.

Ray, B. and Daeschel, M. 1992. Food biopreservatives of microbiological origin. CRC Press, Inc., Boca Raton, FL.

Ray, B. and Hoover, D.G. 1993. Pediocins. In: Hoover, D.G. and Steenson, L.R., (Ed.), Bacteriocins of lactic acid bacteria. Academic Press, Inc., San Diego, CA, pp.181-210.

Reis, M., Eschbach-Bludau, M., Iglesias-Wind, M.I., Kupke, T. and Sahl, H.-G. 1994. Producer immunity towards the lantibiotic Pep5: Identification of the immunity gene *pepI* and localization and functional analysis of its gene product. Applied and Environmental Microbiology 60:2876-2883.

Reisinger, P., Seidel, H., Tschesch, H. and Hammes, W.P. 1980. The effect of nisin on murein synthesis. Archives in Microbiology 37:57-94.

Rhodehamel, E.J. 1992. FDA's concerns with sous vide processing. Food Technology 46(12):73-76.

Roller, S. 1995. The quest for natural antimicrobials as novel means of food preservation: status report on a European research project. International Biodeterioration and Biodegradation 36:333-345.

Salmond, G.P.C., Bycroft, B.W., Stewart, G.S.A.B. and Williams, P. 1995. The bacterial 'enigma': cracking the code of cell-cell communication. Molecular Microbiology 16:615-624.

Saucier, L., Poon, A. and Stiles, M.E. 1995. Induction of bacteriocin production in *Carnobacterium piscicola* LV17. Journal of Applied Bacteriology 78:684-690.

Schillinger, U. and Holzapfel, W.H. 1990. Antibacterial activity of carnobacteria. Food Microbiology 7:305-310.

Schillinger, U. and Holzapfel, W.H. 1995. The genus *Carnobacterium*. In: Wood, B.J.B. and Holzapfel, W.H. (Ed.). The genera of lactic acid bacteria. Blackie Academic and Professional, London, pp. 307-326.

Schillinger, U., Stiles, M.E. and Holzapfel, W.H. 1993. Bacteriocin production by *Carnobacterium piscicola* LV61. International Journal of Food Microbiology 20:131-147.

Schleifer, K.H. and Ludwig, W. 1995. Phylogenetic relationships of lactic acid bacteria. In: Wood, B.J.B. and Holzapfel, W.H. (Ed.). The genera of lactic acid bacteria. Blackie Academic and Professional, London, pp. 7-18.

Schofield, G.M. 1992. Emerging foodborne pathogens and their significance in chilled foods. Journal of Applied Bacteriology 72:267-273.

Shaw, B.G. and Harding, C.D. 1984. A numerical taxonomic study of lactic acid bacteria from vacuum-packed beef, pork, lamb and bacon. Journal of Applied Bacteriology 56:25-40.

Shaw, B.G. and Harding, C.D. 1985. Atypical lactobacilli from vacuum-packaged meats: comparison by DNA hybridization, cell composition and biochemical tests with a description of *Lactobacillus carnis* sp. nov. Systematics in Applied Microbiology 6:291-297.

Shefet, S.M., Sheldon, B.W. and Klaenammer T.R. 1995. Efficacy of optimized nisin-based treatments to inhibit *Salmonella typhimurium* and extend shelf life of broiler carcasses. Journal of Food Protection 58:1077-1082.

Siegers, K. and Entian, K.D. 1995. Genes involved in immunity to the lantibiotic nisin produced by *Lactococcus lactis* 6F3. Applied and Environmental Microbiology 61:1082-1089.

Spencer, J.L., Modler, H.W., Chambers, J.R. and Garcia, M.M. 1993. Proceedings of the Workshop on Lactic Acid Bacteria Research in Canada, Canadian Agri-Food Research Council, Edmonton, AB, pp. 22-42.

Stevens, K.A., Klapes, N.A., Sheldon, B.W. and Klaenhammer T.R. 1992a. Antimicrobial action of nisin against *Salmonella typhimurium* lipopolysaccharide mutants. Applied and Environmental Microbiology 58:1786-1788.

Stevens, K.A., Sheldon, B.W., Klapes, N.A. and Klaenammer T.R. 1991. Nisin treatment for inactivation of *Salmonella* species and other gram-negative bacteria. Applied and Environmental Microbiology 57:3613-3615.

Stevens, K.A., Sheldon, B.W., Klapes, N.A. and Klaenammer T.R. 1992b. Effect of treatment conditions on nisin inactivation of gram-negative bacteria. Journal of Food Protection 55:763-766.

Stiles, M.E. 1991a. Scientific principles of controlled/modified atmosphere packaging. In: Ooraikul, B. and Stiles, M.E. Modified atmosphere packaging of food. Ellis Horwood Ltd., Chichester, England, pp. 18-25.

Stiles, M.E. 1991b. Modified atmosphere packaging of meat, poultry and their products. In: Ooraikul, B. and Stiles, M.E. Modified atmosphere packaging of food. Ellis Horwood Ltd., Chichester, England, pp. 118-147.

Stiles, M.E. 1994. Potential for biological control of agents of foodborne disease. Food Research International 27:245-250.

Stoddard, G.W., Petzel, J.P., van Belkum, M.J., Kok, J. and McKay, L.L. 1992. Molecular analysis of the lactococcin A gene cluster from *Lactococcus lactis* subsp. *lactis* biovar. diacetylactis WM4. Applied and Environmental Microbiology 58:1952-1961.

Stoffels, G., Nissen-Meyer, J., Gudmundsdottir, A., Sletten, K., Holo, H. and Nes, I.F. 1992. Purification and characterization of a new bacteriocin isolated from a *Carnobacterium* sp. Applied and Environmental Microbiology 58:1417-1422.

Tagg, J.R., Dajani, A.S. and Wannamaker, L.W. 1976. Bacteriocins of gram-positive bacteria. Bacteriological Reviews 40:722-756.

Thorne, S. 1986. The history of food preservation. Barnes and Nobles Books, Totowa, NJ.

Thornley, M.J. 1957. Observations on the microflora of minced chicken meat irradiated with 4 MeV cathode rays. Journal of Applied Bacteriology 20:286-298.

Thornley, M.J. and Sharpe, M.E. 1959. Micro-organisms from chicken meat related to both lactobacilli and aerobic sporeformers. Journal of Applied Bacteriology 22:368-376.

Todd, E.C.D. 1989. Costs of acute bacterial foodborne disease in Canada and the United States. International Journal of Food Microbiology 9:313-326.

Todd, E.C.D. 1992. Foodborne disease in Canada - a 10-year summary from 1975 to 1984. Journal of Food Protection 55:123-132.

Todd, E.C.D. 1994. Surveillance of foodborne disease. In: Hui, Y.H., Gorham, J.R., Murrell, K.D. and Cliver, D.O. (Ed.), Foodborne disease handbook: diseases caused by bacteria. Marcel Dekker, Inc., New York, NY. pp. 461-536.

Todd, E.C.D. and Harwig, J. 1996. Microbial risk analysis of food in Canada. Journal of Food Protection, 59 suppl.:10-18.

Tomita, H., Fujimoto, S., Tanimoto, K. and Ike, Y. 1996. Cloning and genetic organization of the bacteriocin 31 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pY117. Journal of Bacteriology 178:3585-3593.

Tortora, G.J., Funke, B.R. and Case, C.L. 1992. Microbiology: an introduction. The Benjamin/Cummings Publishing Company, Inc., Redwood City, CA.

Toussaint-Samat, M. 1994. History of food. Translated by Anthea Bell. Blackwell Publishers, Cambridge, MA.

Urbain, W.M. and Campbell, J.F. 1987. Meat preservation. In: Price, J.F. and Schweigert, B.S. (Ed.), The science of meat and meat products, 3rd ed. Food and Nutrition Press, Inc., Westport, CN, pp. 371-412.

Vandamme, P., Pot, P., Gillis, M., De Vos, P., Kersters, K. and Swings, J. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiological Reviews 60:407-438.

Van der Meer, J.R., Polman, J., Beerthuyzen, M.M., Siezen, R.J., Kuipers, O.P. and de Vos, W.M. 1993. Characterization of the *Lactococcus lactis* nisin A operon genes *nisP*, encoding a subtilisin-like serine protease involved in precursor processing, and *nisR*, encoding a regulatory protein involved in nisin biosynthesis. Journal of Bacteriology 175:2578-2588.

Vining, L.C. 1992. Roles of secondary metabolites from microbes. In: Chadwick, D.J. and Whelan, J. (Ed.), Secondary metabolites: their function and evolution. Ciba Foundation Symposium 171. John Wiley and Sons, Chichester, England, pp. 184-198.

Wan, J., Hickey, M.W. and Coventry, M.J. 1995. Continuous production of bacteriocins, brevicin, nisin and pediocin, using calcium alginate-immobilized bacteria. Journal of Applied Bacteriology 79:671-679.

West, C.A. and Warner, P.J. 1988. Plantacin B, a bacteriocin produced by *Lactobacillus plantarum* NCDO 1193. FEMS Microbiology Letters 49:163-165.

Worobo, R.W. 1996. Characterization of two bacteriocins and a food grade plasmid from carnobacteria. Ph.D. thesis, University of Alberta, Edmonton, AB.

Worobo, R.W., Henkel, T., M., Roy, K.L., Vederas, J.C. and Stiles, M.E. 1994. Characteristics and genetic determinant of a hydrophobic peptide bacteriocin, carnobacteriocin A, produced by *Carnobacterium piscicola* LV17A. Microbiology (UK) 140:517-526.

Worobo, R.W., Van Belkum, M.J., Sailer, M., Roy, K.L., Vederas, J.C. and Stiles, M.E. 1995. A signal peptide secretion-dependent bacteriocin from *Carnobacterium divergens*. Journal of Bacteriology 177:3143-3149.

"Ever since the Great Famine, the Irish have lived by the rules of a post-fungus culture, keeping their numbers steady and constant... Phytophthora infestans and Rickettsia are as much a part of Irish culture as fairies".

The fourth horseman
Andrew Nikiforuk

Chapter 2

Induction of Bacteriocin Production in *Carnobacterium piscicola* LV17

A version of this chapter has been published. Saucier, L., Poon., A. and Stiles, M.E. 1995. Journal of Applied Bacteriology 78:684-690.

2.1. Introduction

Bacteriocin production by lactic acid bacteria is being studied for use in biopreservation of foods that support the growth of lactic acid bacteria as their prevailing microflora. A wide range of lactic acid bacteria has been associated with chill stored meats that are packaged under vacuum or in modified atmospheres with elevated levels of carbon dioxide (Shaw and Harding, 1984; McMullen and Stiles, 1993). Among these, a new genus *Carnobacterium* has been described (Collins *et al.*, 1987) and strains that produce bacteriocins have been reported (Ahn and Stiles, 1990a; Schillinger and Holzapfel, 1990; Stoffels *et al.*, 1992). The bacteriocinogenic activity of *Carnobacterium piscicola* LV17 could be of special interest because its bacteriocins are active against a wide range of lactic acid bacteria and strains of *Listeria* and *Enterococcus* (Ahn and Stiles, 1990a), and they are detected early in the growth cycle when inoculated at 10^7 colony forming units (cfu) per ml in synthetic medium (Ahn and Stiles, 1990b).

An early review indicated that production of bacteriocins by various organisms was not constitutive (Reeves, 1965) and Meghrous *et al.* (1992) demonstrated that nisin production is tightly regulated with growth rate and composition of the growth medium. Furthermore, De Vuyst and Vandamme (1992) showed that carbon source plays a major role in nisin production. Geis *et al.* (1983) observed that 65 strains (23%) of lactic streptococci showed antagonism on solid medium but only 16 strains (6%) produced detectable antagonistic substances in liquid medium. De Klerk (1967) reported that growth on solid media increased the incidence of bacteriocinogenicity in *Lactobacillus fermenti.* Some strains only produce bacteriocin on solid media, as reported for

lactacin B from *Lactobacillus acidophilus* and plantacin B from *Lactobacillus plantarum* (Barefoot and Klaenhammer, 1983; West and Warner, 1988).

It was while determining the inoculum size of the bacteriocin-producing strain, *C. piscicola* LV17, necessary to inhibit the growth of the target strain, *C. divergens* LV13, that bacteriocin production was found to be dependent on the level of inoculation. The results obtained demonstrated that *C. piscicola* LV17 rapidly dominated *C. divergens* LV13 when inoculated at 10^7 cfu/ml. In contrast, when both strains were inoculated at 10^5 or 10^3 cfu/ml, the target strain grew as well as *C. piscicola* LV17, the bacteriocin producer. It was subsequently shown that bacteriocin production by *C. piscicola* LV17 always occurred in liquid medium with an inoculum of 10^7 cfu/ml; bacteriocin production was unpredictable at 10^5 cfu/ml; but bacteriocin was not detected with an inoculum of 10^4 cfu/ml or lower. When a fully grown noninduced Bac^- culture of *C. piscicola* LV17 was subsequently diluted at $\geq 10^6$ cfu/ml and incubated until full growth was reached, bacteriocin production was not detected in the supernatant. Bacteriocin production in a fully grown noninduced Bac^-culture of *C. piscicola* LV17 was only restored when the culture was streaked onto APT agar medium (Saucier *et al.*, 1995). This chapter reports the induction of bacteriocin production from a low inoculum ($< 10^4$ cfu/ml) and the presence of an inducing factor for bacteriocin production in the supernatant of *C. piscicola* LV17.

2.2. Materials and Methods

2.2.1. Bacterial cultures

The bacteriocin producing strain *C. piscicola* [proposed name *C. maltaromicus,* Collins *et al.* (1991)] LV17 and the target strain *Carnobacterium*

divergens LV13 were originally obtained from Dr. B.G. Shaw (Institute of Food Research, Langford, Bristol, U.K.; cultures are available from the National Collection of Food Bacteria as strains 2852 and 2855, respectively). The producer strain was cured of its plasmids and modified to contain separate plasmids responsible for bacteriocin production (Ahn and Stiles, 1992). Strains used in this study are listed in Table 2-1. Cultures were grown for 24 h unless otherwise specified and plates were incubated anaerobically (10% CO_2, 90% N_2) at room temperature (25°C) for 2 or more days. Stock cultures were prepared by centrifugation (10000 x *g*, 15 min) of a fully grown culture in APT broth (All Purpose Tween; BBL, Becton-Dickinson Microbiology Systems, Cockeysville, MD), resuspended in half of the original volume in sterile APT broth containing 20% glycerol (vol./vol.) and stored at -70°C. Cultures for use in experiments were subcultured directly from frozen stock cultures into APT broth. Subcultures (3%) in APT were incubated at room temperature (25°C) for 24 h for at least two, but not more than seven, successive daily subcultures; all subcultures and experiments were done in 6 ml volume.

2.2.2. Assay for bacteriocin activity

Inhibitory activity was screened by direct and deferred antagonism techniques and assayed by the spot-on-lawn technique using heat-treated supernatant (65°C for 30 min) on a lawn of *C. divergens* LV13. Bacteriocin activity was expressed in arbitraty activity units (AU) per ml based on the reciprocal of the lowest of a series of dilutions that displayed complete inhibition of a target organism, *C. divergens* LV13 (Ahn and Stiles, 1990a, b; Hoover and Harlander, 1993).

Table 2-1. Organisms used in this study

Strains [a]		Description [b]	Reference
Carnobacterium piscicola	LV17	wild-type, native plasmids pCP9, pCP40, pCP49 CbnA$^+$, CbnBM1$^+$, CbnB2$^+$, CbnA^r, CbnBM1^r, CbnB2^r	Shaw [c]
	LV17A	CbnA$^+$, CbnA^r, CbnB^s, native plasmid pCP49	Ahn and Stiles, 1992
	LV17B	CbnBM1$^+$, CbnB2$^+$, CbnBM1^r, CbnB2^r, CbnA^s, native plasmid pCP40	Ahn and Stiles, 1992
	LV17C	Bac$^-$ phenotype, CbnA^s, CbnB^s, plasmidless	Ahn and Stiles, 1992
	LV17C/pLQ24	LV17C cloned with pLQ24 (CbnB2$^+$, CbnB2^r), 16-kb *Pst*I fragment of pCP40	Quadri *et al.*, 1995
	LV17C/pLQ18	LV17C cloned with pLQ18 (CbnB2$^+$, CbnB2^r), 9.5-kb *Pst*I fragment of pCP40	Quadri *et al.*, 1995
	UAL26	Bac$^+$, plasmidless	Burns, 1989[c]
	UAL26/pCP49	Bac$^+$, UAL 26 cloned with pCP49 (CbnA$^+$, CbnA^r)	Ahn and Stiles, 1992
	UAL26/pLQ24	Bac$^+$, UAL 26 cloned with pLQ24 (CbnB2$^+$, CbnB2^r)	Quadri *et al.*, 1995
Carnobacterium divergens	LV13	Dvn$^+$, Dvn^r native plasmid pCD3.4	Worobo *et al.*, 1995
	LV13/pLQ24	Dvn$^+$, Dvn^r native plasmid pCD3.4 and pLQ24 (CbnB2$^+$, CbnB2^r)	Quadri *et al.*, 1995
Leuconostoc gelidum	UAL187	wild-type, native plasmids pLG5.0, pLG7.6 and pLG9.2	Hastings *et al.*, 1991
	UAL187.22	Lcn$^+$, Lcn^r, native plasmids pLG 7.6 and pLG9.2	Hastings *et al.*, 1991
	UAL187.13	Lcn$^-$, Lcn^s, native plasmid pLG 9.2	Hastings *et al.*, 1991
Lactococcus lactis ssp. *lactis*	ATCC 11454	Nis$^+$, Nis^r	
	UAL 245	Bac$^+$, isolated from vacuum packed beef	Mishra, 1992
	UAL 276	Bac$^+$, isolated from vacuum packed beef	Mishra, 1992

a ATCC = American Type Culture Collection, UAL = University of Alberta Lactic Acid Bacteria collection.

b Bac = bacteriocin (unnamed); Cbn = carnobacteriocin; Lcn = leucocin A-UAL187, Dvn = divergicin, Nis = nisin, r = resistant, s = sensitive.

c Supplied by Dr. B.G. Shaw, Institute for Food Research, Langford, Bristol, UK.

2.2.3. Induction studies

Supernatant (500 µl) from an overnight culture was heated in a boiling water bath for 15 min to kill the viable cells before addition of 1% (vol./vol.; now called induction solution) to a 10^4 cfu/ml culture, unless otherwise specified. The proteinaceous nature of the inducing compound in the heat-treated supernatant was determined with pronase E (Sigma, St. Louis, MO; 0.1 mg/ml, 37°C for 60 min). The pronase was inactivated in a boiling water bath for 15 min prior to addition to a 10^4 cfu/ml culture. As an alternative to heat-treatment of the supernatant, a low protein binding filter (Acrodisc®, pore size 0.2 µm, Gelman Sciences, Ann Arbor, MI) was used to sterilize the bacteriocin-containing supernatant. Carnobacteriocins A1, A2, A3, B1, BM1 and B2 were purified as reported by Sailer *et al.* (1993), Quadri *et al.* (1994) and Worobo *et al.* (1994). The first step consisted of a separation by hydrophobic affinity chromatography (Amberlite XAD-8, BDH Chemicals, Toronto) using 0.1% trifluoroacetic acid (TFA) followed by step-wise elution with ethanol (20 to 90%). After concentration by rotary evaporation at 30°C, active fractions were applied to a Sephadex LH-60 column using acetonitrile as the eluent. Purification to homogeneity was obtained using a C_8 HPLC column. The pure bacteriocins used to induce bacteriocin production at low inoculum of 10^4 cfu/ml were supplied by Dr. M. Sailer and Dr. J.C. Vederas. The bacteriocins were resuspended at various concentrations in 0.1% TFA and tested for induction of bacteriocin production in a culture containing 10^4 cfu/ml. Background activity from the bacteriocin added to induce production was taken into account in determining induction at high concentrations.

2.3. Results

While determining the inoculum size of *C. piscicola* LV17 necessary to inhibit the growth of the target strain, *C. divergens* LV13, it was discovered that bacteriocin production was dependent on the size of inoculum (Saucier *et al.*, 1995). Although bacteriocin production occurs only with an inoculum $\geq 10^6$ cfu/ml, similar growth rates and maximum populations were observed for all levels of inoculation. Subculturing of a producer strain (1% in APT broth) with partial activity (< 800 AU/ml, maximum produced by a Bac^+ culture) resulted in full bacteriocin production (data not shown). None of the other bacteriocin-producing strains listed in Table 2-1 (UAL26, UAL187, UAL187.22, UAL245 and UAL276) was dependent on inoculum size for bacteriocin production, except for the genetic variants LV17A and LV17B of the wild-type producer strain. Results presented in Table 2-2 demonstrate that bacteriocin production can be restored in a noninduced Bac^- culture by addition of heat-treated supernatant from a Bac^+ fully grown culture, provided that it was added prior to the stationary phase of growth. Bacteriocin activity was not detected (< 50 AU/ml) in a 1% dilution of the heat-treated supernatant. Furthermore, the data in Fig. 2-1 demonstrate that bacteriocin production can only be detected when the cell density reached > 10^7 cfu/ml in both the high inoculum culture (10^7 cfu/ml) and the low inoculum culture (10^4 cfu/ml) induced for bacteriocin production by addition of heat-treated supernatant.

Based on the observation that addition of heat-treated supernatant of a Bac^+ culture of *C. piscicola* LV17 induced bacteriocin production in a noninduced Bac^- culture, experiments were designed to determine the nature of the inducing factor (Table 2-3). Viable cells were removed by filter sterilization

Table 2-2. Bacteriocin production [a] by *C. piscicola* LV17 in APT broth subcultured at different inoculation levels and induced for bacteriocin production by addition of heat treated supernatant fluids

Time of induction [b] (h)	Inoculation level (cfu/ml)		
	10^7	10^5	10^3
	Bacteriocin production (AU/ml)		
0	800	800	800
6	800	800	800
12	800	800	800
18	[c] → 800	800	800
21	800	→ 200	800
24	ND [d]	200	400
27	ND	200	→ <50
30	ND	200	<50

[a] Bacteriocin production was assayed after 36 h of incubation when cultures were fully grown.

[b] Corresponds to the time at which the heat treated supernatant fluids were added to the growing culture.

[c] → indicates the time at which maximum population (stationary phase) was reached.

[d] ND = not determined.

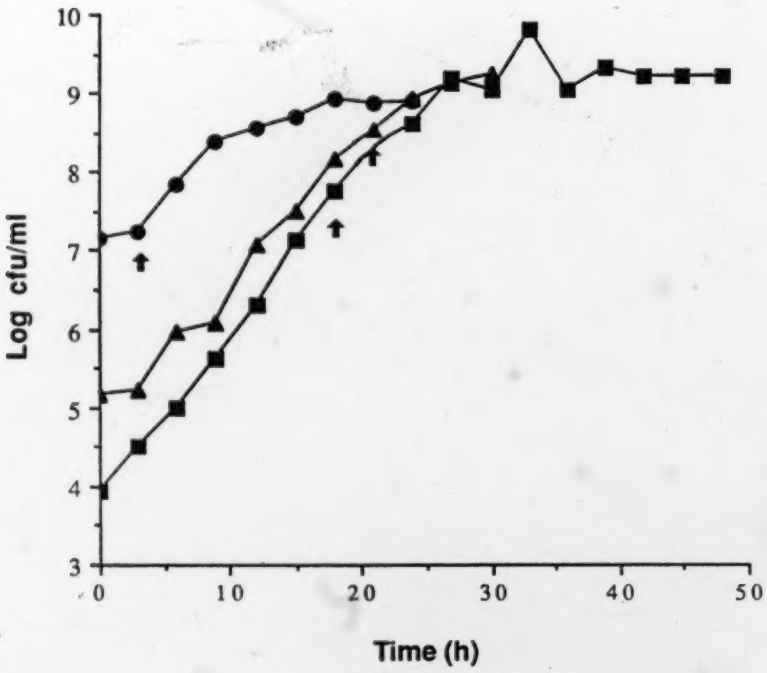


Fig. 2-1. Time course experiment of growth and detection of bacteriocin production by *C. piscicola* LV17 inoculated at 10^7 (●), 10^5 (▲) or 10^4 (■) cfu/ml. The culture containing 10^4 cfu/ml was induced for bacteriocin production by addition of 1% heat treated supernatant of a fully grown positive culture at time zero. The arrows indicate when bacteriocin started to be detected in the supernatant.

Table 2-3. Bacteriocin production by *C. piscicola* LV17, LV17A and LV17B subcultured at 10^7 or 10^4 cfu/ml in APT broth with or without addition of 1% of heat treated supernatant fluids [a]

Producer strains	Inoculation level (cfu/ml)	Induction solution	Bac production (AU/ml)
C. piscicola LV17	10^7	no addition (positive control)	800
	10^4	no addition (negative control)	neg [b]
	10^4	HTS [c] from LV17 (Bac^+)	800
	10^4	filter sterilized supernatant fluids from LV17 (Bac^+)	800
	10^4	HTS from LV17 (Bac^-)	neg
	10^4	Pronase E treated HTS from LV17 (Bac^+)	neg
	10^4	HTS from LV17A	800
	10^4	HTS from LV17B	800
	10^4	HTS from LV17C	neg
	10^4	HTS from LV17C/pLQ24	800
	10^4	HTS from LV17C/pLQ18	800
	10^4	HTS from UAL26	neg
	10^4	HTS from UAL26/pCP49	800
	10^4	HTS from UAL26/pLQ24	800
	10^4	HTS from LV13	neg
	10^4	HTS from LV13/pLQ24	800
C. piscicola LV17A	10^7	no addition (positive control)	800
	10^4	no addition (negative control)	neg
	10^4	HTS from LV17A	800
	10^4	HTS from LV17B	800
	10^4	HTS from LV17C	neg
C. piscicola LV17B	10^7	no addition (positive control)	200
	10^4	no addition (negative control)	neg
	10^4	HTS from LV17B	200
	10^4	HTS from LV17A	200
	10^4	HTS from LV17C	neg

[a] Strains tested are described in Table 2-1. Supernatant was heated at 100°C for 15 min.

[b] neg = negative (> 50 AU/ml).

[c] HTS = heat treated supernatant.

and it was shown that unheated cell-free supernatant induced bacteriocin production in a culture containing 10^4 cfu/ml. Neither the heat-treated supernatant of a fully grown Bac$^-$ culture of *C. piscicola* LV17C nor the heat-treated supernatant of a fully grown Bac$^+$ culture treated with Pronase E induced bacteriocin production in *C. piscicola* inoculated at 10^4 cfu/ml (Table 2-3).

Induction of bacteriocin production by *C. piscicola* LV17 only occurred with addition of supernatant of the wild type strain and from genetic variants producing either carnobacteriocin A, carnobacteriocins B2 or B2 and BM1 (i.e., LV17A, LV17B, LV17C/pLQ24, LV17C/pLQ18, UAL26/pCP49, UAL26/pLQ24 and LV13/pLQ24) and was not induced with UAL187.13 (Lcn$^-$; Table 2-3). To evaluate if bacteriocin induction coincides with bacteriocin production in either *C. piscicola* LV17A or LV17B, addition of heat-treated supernatant fluids (1%) taken at different time intervals during growth was used to induce bacteriocin production in *C. piscicola* LV17 inoculated at 10^4 cfu/ml. Bacteriocin induction occurred 1.5 to 3 h before bacteriocin could be detected in the supernatant fluids of *C. piscicola* LV17A or LV17B using the spot-on-lawn technique (Table 2-2). Also, bacteriocin induction in *C. piscicola* by supernatant of *C. piscicola* LV17A occurred 6 h earlier in the growth cycle than with LV17B, corresponding to the earlier production of bacteriocin by LV17A. *C. piscicola* LV17A and LV17B were antagonistic to each other as determined by direct and deferred inhibition (Ahn and Stiles, 1990b); however, bacteriocin production by *C. piscicola* LV17A was induced by the supernatant of LV17B, and vice versa, but achievement of maximum cell population was delayed by up to 24 h (data not shown).

In order to identify the inducing compound, procedures previously reported for bacteriocin purification were used (Sailer *et al.*, 1993; Quadri *et al.*,

1994; Worobo *et al.*, 1994). Supernatant of a Bac$^+$ culture of *C. piscicola* LV17A grown on casamino acids medium was applied to a hydrophobic XAD-2 column (Sailer *et al.*, 1993) and collected fractions were pooled as indicated in Table 2-4. Pooled fractions were tested for bacteriocin induction using a *C. piscicola* LV17 inoculum of 10^4 cfu/ml. Before concentration by evaporation, only the Bac$^+$ pooled fractions III and V induced bacteriocin production. After concentration, pooled fraction VI induced bacteriocin production but to a lower extent compared with pooled fractions III and pooled fractions V. Bacteriocin activity was not detected from concentrated, pooled fractions VI . Solutions of pure bacteriocin obtained from Dr. Vederas' laboratory were tested for their ability to induce bacteriocin production of a 10^4 cfu/ml culture (Table 2-5). The only one of the three active compounds purified from the supernatant of *C. piscicola* LV17A that induced bacteriocin production in *C. piscicola* LV17, LV17A and LV17B to the same level as a culture containing 10^7 cfu/ml of these strains was the sulfoxide derivative of the bacteriocin, carnobacteriocin A2. Carnobacteriocin A1 is a degradation mixture of carnobacteriocin A (Worobo *et al.*, 1994) and gave partial induction of LV17B (50 to 100 AU/ml). *C. piscicola* LV17B produces two distinct bacteriocins designated carnobacteriocins B2 and BM1; carnobacteriocin B1 is a sulfoxide derivative of BM1 (Quadri *et al.*, 1994). All three of the carnobacteriocins produced by *C. piscicola* LV17B induced bacteriocin production of a culture 10^4 cfu of *C. piscicola* LV17 and LV17A per ml; although with carnobacteriocin B1, the level of induction of LV17 was only 200 AU/ml. Only carnobacteriocin B2 induced bacteriocin activity of *C. piscicola* LV17B.

Table 2-4. Bacteriocin production by *C. piscicola* LV17 induced for bacteriocin production by addition of supernatant fluids of *C. piscicola* LV17A separated by hydrophobic affinity chromatography.

Pool	Fraction description [a]	Bacteriocin induction (AU/ml) unconcentrate	concentrate [b]
I	0.1% TFA, Bac^-	neg	neg
II	Ethanol 20 and 40%, Bac^-	neg	neg
III	Ethanol 40%, Bac^+	100	400
IV	Ethanol 40 and 80%, Bac^-	neg	neg
V	Ethanol 80%, Bac^+	100	1600
VI	Ethanol 80 and 90%, Bac^-	neg	50

[a] All ethanol solutions were diluted in 0.1% TFA.

[b] Pooled fractions were concentrated under vacuum to a volume < 5 ml using a rotary evaporation water bath at a temperature < 35°C.

Table 2-5. Bacteriocin production by *C. piscicola* LV17, LV17A and LV17B induced for bacteriocin production by addition of pure carnobacteriocins

Carnobacteriocins	Induction of carnobacteriocin production in *C. piscicola* LV17		
	LV17	LV17A	LV17B
A1 (degradation product of A2 & A3)	−	−	±[a]
A2 (sulfoxide derivative of A3)	+	+	+
A3 (unmodified bacteriocin, pCP49)	−	−	−
B1 (sulfoxide derivative of BM1)	+[a]	+	−
BM1 (unmodified bacteriocin, chromosome)	+	+	−
B2 (unmodified bacteriocin, pCP40)	+	+	+

[a] Bacteriocin production (AU /ml) was 2 to 4 times less than the respective positive control (Table 2-3).

2.4. Discussion

This study indicated that bacteriocin production was not constitutive in *C. piscicola* LV17 but rather dependent on specific conditions. The fact that a noninduced Bac$^-$ culture of *C. piscicola* LV17 did not produce bacteriocin during subsequent subculture at > 0.01% in APT broth, but that addition of 1% of heat-treated supernatant of a Bac$^+$ culture to the growing cells induced bacteriocin production, indicated that a certain concentration of an induction factor was required for bacteriocin production. Complementary action of two peptides has been reported as one of the mechanisms involved in bacteriocin activity (Nes *et al.*, 1996). In this study, the inducing factor was not present in the supernatant of the noninduced Bac$^-$ culture or the Bac$^-$ plasmidless strain LV17C indicating that the complementary action of two peptides obtained from different fractions, as previously described for maximum activity, does not explain the dependence of bacteriocin production on inoculation level . On the contrary, the active compound has to be present during exponential growth for induction to occur.

Barefoot *et al.* (1994) reported that associative growth of an indicator strain, *Lactobacillus delbrueckii* ssp. *lactis* ATCC 4797, with the lactacin B producer *Lactobacillus acidophilus* N2 increased lactacin B production. An intracellular protein from the indicator strain induced lactacin B production. With *C. piscicola* LV17, associative growth of the indicator, *C. divergens* LV13, did not induce bacteriocin production by the producer strain (Saucier *et al.*, 1995) and the heat-treated supernatant of the indicator strain did not induce bacteriocin production in a Bac$^-$ inoculum of *C. piscicola* LV17. Only the supernatant of genetic variants that encode carnobacteriocin production induced bacteriocin production in a Bac$^-$ inoculum of *C. piscicola* LV17 and, using purified

carnobacteriocins, it appeared that bacteriocins produced by *C. piscicola* LV17 regulated the expression of the Bac$^+$ phenotype. This particular phenomenon fits the description of autoregulation (Goldberger, 1974; Maloy and Stewart, 1993). Of the two active compounds purified from the supernatant of *C. piscicola* LV17A, it was the sulfoxide homologue A2, and not the unoxidized carnobacteriocin A3, that induced bacteriocin production by *C. piscicola* LV17, LV17A and LV17B. Methionine at position 52 of carnobacteriocin A2 is considered to be oxidized as a sulfoxide (Worobo *et al.*, 1994). Sulfoxide residues produced by oxidation of methionine have been reported for various proteins with and without loss of their biological activity (Brot and Weissbach 1983; Pearlman and Nguyen 1992). Methionine residues may be oxidized during the purification process (Kimmel *et al.*, 1984). In recombinant human interleukin 2, the methionine residues were spontaneously oxidized when it was stored for long periods in aqueous solution (Sasaoki *et al.*, 1989). It seems unlikely that an oxidation product would have a biological regulatory function; however, carnobacteriocin B1 is the sulfoxide homologue of carnobacteriocin BM1 and it was suggested by Quadri *et al.* (1994) that the oxidized form is produced during growth of the producer strain. The inducer of sakacin P was also in its oxidized form when it was purified from the supernatant of the producer strain. However, this oxidized state is not essential for induction of bacteriocin as demonstrated with the synthetic peptide which was not oxidized (Eijsink *et al.*, 1996). In this study the unoxidixed carnobacteriocin A3 did not induce bacteriocin production.

It is interesting to note that when the bacteriocin determinants are cloned into the pCaT expression vector and transformed into homologous or heterologous hosts (LV17C/pLQ24, LV17C/pLQ18, UAL26/pCP49, LV13/pLQ24)

or as the original plasmid in an heterologous host (UAL26/pCP49), bacteriocin production becomes a constitutive feature under conditions where bacteriocin production by *C. piscicola* LV17 is dependent of the inoculum size. Unfortunately the copy number of pCaT expression vector is not known (Ahn *et al.*, 1992). If it is a high copy number plasmid, it could be postulated that a repressor gets titrated out. Alternatively, the repressor may not be produced in an heterologous host containing pCP49 (UAL26/pCP49) In bacteriocin-producing heterologous hosts, such as *C. divergens* LV13 and *C. piscicola* UAL26, the original bacteriocin production systems might overcome the regulation of bacteriocin production related to the size of the inoculum.

Bacteriocin production reached a maximum of 800 AU/ml with an inoculum of $\geq 10^6$ cfu/ml, was variable at 10^5 cfu/ml, and it was lost at $\leq 10^4$ cfu/ml. A fully grown Bac^+ culture of 10^8 to 10^9 cfu/ml diluted down to 10^5 to 10^6 cfu/ml will have a carnobacteriocin concentration of 1 AU/ml which is therefore the required bacteriocin concentration for induction. Full bacteriocin production could be induced from a 10^5 cfu/ml culture that exhibited an intermediate production of bacteriocin when tested as follows: samples taken at different times during the growth cycle of a 10^5 cfu/ml culture, diluted 1:100 and incubated until fully grown, achieved maximum production of bacteriocin when the bacteriocin concentration in the diluted culture reached 1 AU/ml. Furthermore, addition of > 0.01% supernatant to a Bac^- inoculum of *C. piscicola* LV17 was necessary to get the same bacteriocin production as the Bac^+ culture containing 10^7 cfu/ml; a concentration of 0.01% supernatant is equivalent to dilution at 10^5 cfu/ml. However, cell density needed to reach a certain threshold before bacteriocin could be detected indicating that a critical ratio of inducer to cell population might serve as a signal for bacteriocin production. Bacterial

communication triggered by sensing the cell density has been reported and it is referred to as "quorum sensing." Such a system allows the organism to optimize different behaviors when the population has reached a certain threshold as in the case for bioluminescence in *Photobacterium fischeri* and streptomycin production and sporulation in *Streptomyces griseus*. In these examples the inducer is an homoserine lactone molecule (Fuqua *et al.*, 1994). In *C. piscicola* LV17, the bacteriocins are autoregulated but they are not the molecules responsible for quorum sensing as described in Fuqua *et al.* (1994). With all inocula tested, bacteriocin production did not occur with a $\leq 10^4$ cfu/ml inoculum, even if maximum cell population is reached.

A similar inducer to cell density relationship might account for the recovery of bacteriocin production on solid media. In agar, the active compound would not diffuse as readily as in solution, allowing the cells on agar to be in closer contact with more of the active compound than in liquid medium. The same dependence on the level of inoculation for bacteriocin production was also reported in *Lactobacillus plantarum* and *Lactobacillus sake* but recovery of bacteriocin production from a noninduced Bac^- culture by growth on solid medium was not possible, indicating that the mechanisms of regulation differ from that for *C. piscicola* LV17 (Diep *et al.*, 1995; Eijsink *et al.*, 1996). Differences in bacteriocin production between media have been observed, especially between initial screening on solid media and subsequent growth in liquid media (De Klerk, 1967; Geis *et al.*, 1983). Composition of growth medium and pH were also shown to influence bacteriocin production by lactic acid bacteria (Kozak and Dobrzanski, 1977; Kaiser and Montville, 1993) and similar observations were reported for *C. piscicola* LV17 by Ahn and Stiles (1990a). When bacteriocinogenic strains are studied in a food system for use as

biopreservatives, it cannot be assumed that the Bac$^+$ phenotype will be expressed because it may not be a constitutive trait. Means to evaluate the expression of Bac$^+$ phenotype in the system under study are imperative to assess the real value of a bacteriocin as a biopreservative.

Using pure bacteriocins, we demonstrated that bacteriocins of *C. piscicola* LV17 exerted a positive autoregulation on the Bac$^+$ phenotype. *C. piscicola* LV17A bacteriocin induced production by *C. piscicola* LV17B, and vice versa, but depending on the amount (activity) of the bacteriocin added, delay of growth of the producer strain for 24 h or more was noted. In this case, resistance might need to develop before the challenged cells are induced. The mechanism whereby induction takes place is not known, but messenger RNA studies in Chapter 3 indicate that regulation of induction occurs at the level of transcription of the bacteriocin structural gene.

2.5. References

Ahn, C. and Stiles, M.E. 1990a. Antibacterial activity of lactic acid bacteria isolated from vacuum-packaged meats. Journal of Applied Bacteriology 69:302-310.

Ahn, C. and Stiles, M.E. 1990b. Plasmid-associated bacteriocin production by a strain of *Carnobacterium piscicola* from meat. Applied and Environmental Microbiology 56:2503-2510.

Ahn, C. and Stiles, M.E. 1992. Mobilization and expression of bacteriocin plasmids from *Carnobacterium piscicola* isolated from meat. Journal of Applied Bacteriology 73:217-228.

Ahn, C., Collins-Thompson, D., Duncan, C. and Stiles, M.E. 1992. Mobilization and location of the genetic determinant of chloramphenicol resistance from *Lactobacillus plantarum* caTC2R. Plasmid 27:169-176.

Barefoot, S.F. and Klaenhammer, T.R. 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Applied and Environmental Microbiology 45:1808-1815.

Barefoot, S.F., Chen, Y.-R., Hughes, T.A., Bodine, A.B., Shearer, M.Y. and Hughes, M.D. 1994. Identification and purification of a protein that induces production of the *Lactobacillus acidophilus* bacteriocin lactacin B. Applied and Environmental Microbiology 60:3522-3528.

Brot, N. and Weissbach, H. 1983. Biochemistry and physiological role of methionine sulfoxide residues in proteins. Archives of Biochemistry and Biophysics 223:271-281.

Burns, K.A. 1987. Potential of a lactic acid bacterium for the biological preservation of ground beef. M.Sc. thesis, University of Alberta, Edmonton, AB.

Collins, M.D., Farrow, J.A.E., Phillips, B.A., Ferusu, S. and Jones, D. 1987. Classification of *Lactobacillus divergens*, *Lactobacillus piscicola*, and some catalase-negative, asporogenous, rod-shaped bacteria from poultry in a new genus, *Carnobacterium*. International Journal of Systematic Bacteriology 37:310-316.

Collins, M.D., Rodrigues, U., Ash, C., Aguirre, M., Farrow, J.A.E., Martinez-Murcia, A., Phillips, B.A., Williams, A.M. and Wallbanks, S. 1991. Phylogenetic analysis of the genus *Lacobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. FEMS Microbiology Letters 77:5-12.

De Klerk, H.C. 1967. Bacteriocinogeny in *Lactobacillus fermenti*. Nature 214:609.

De Vuyst, L. and Vandamme, E.J. 1992. Influence of the carbon source on nisin production in *Lactococcus lactis* subsp. *lactis* batch fermentations. Journal of General Microbiology 138:571-578.

Diep, D.B., Håvarstein, L.S. and Nes, I.F. 1995. A bacteriocin-like peptide induces bacteriocin synthesis in *Lactobacillus plantarum* C11. Molecular Microbiology 18:631-639.

Eijsink, V.G.H., Brurberg, M.B., Middelhoven, P.H. and Nes, I.F. 1996. Induction of bacteriocin production in *Lactobacillus sake* by a secreted peptide. Journal of Bacteriology 178:2232-2237.

Fuqua, W.C., Winans, S.C. and Greenberg, E.P. 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. Journal of Bacteriology 176:269-275.

Geis, A., Singh, J. and Teuber, M. 1983. Potential of lactic streptococci to produce bacteriocin. Applied and Environmental Microbiology 45:205-211.

Goldberger, R.F. 1974. Autogenous regulation of gene repression. Science 183:810-816.

Hastings, J.W., Sailer, M., Johnson, K., Roy, K.L., Vederas, J.C. and Stiles, M.E. 1991. Characterization of leucocin A-UAL 187 and cloning of the bacteriocin gene from *Leuconostoc gelidum*. Journal of Bacteriology 173:7491-7500.

Hoover, D.G. and Harlander, S.K. 1993. Screening methods for detecting bacteriocin activity. In: Hoover, D.G. and Steenson, L.R. (Ed.), Bacteriocins of lactic acid bacteria. Academic Press, Inc., San Diego, CA, pp. 23-39.

Kaiser, A.L. and Montville, T.J. 1993. The influence of pH and growth rate on production of the bacteriocin, bavaricin MN, in batch and continuous fermentations. Journal of Applied Bacteriology 75:536-540.

Kimmel, J.R., Pollock, H.G., Chance, R.E., Johnson, M.G., Reeve, J.R., Taylor, I.L., Miller, C. and Shively, J.E. 1984. Pancreatic polypeptide from rat pancreas. Endocrinology 114:1725-1731.

Kozak, W. and Dobrzanski, W.T. 1977. Growth requirements and the effect of organic components of the synthetic medium on the biosynthesis of the antibiotic nisin in *Streptococcus lactis* strain. Acta Microbiologica Polonica 26:361-368.

Maloy, S. and Stewart, V. 1993. Autogenous regulation of gene expression. Journal of Bacteriology 175:307-316.

McMullen, L.M. and Stiles, M.E. 1993. Microbial ecology of fresh pork stored under modified atmosphere at -1, 4.4 and 10°C. International Journal of Food Microbiology 18:1-14.

Meghrous, J., Huot, E., Quittelier and Petitdemange, H. 1992. Regulation of nisin biosynthesis by continuous cultures and by resting cells of *Lactococccus lactis* subsp. *lactis*. Research in Microbiology 143:879-890.

Mishra, C.V. 1992. Characterization of broad spectrum bacteriocins produced by lactic acid bacteria isolated from vacuum packaged raw beef. M.Sc. thesis, University of Alberta, Edmonton, AB.

Nes, I.F., Diep, D.B., Håvarstein, L.S., Brurberg, M.B., Eijsink, V. and Holo, H. 1996. Biosynthesis of bacteriocins in lactic acid bacteria. Antonie van Leeuwenhoek 70:113-128.

Pearlman, R. and Nguyen, T. 1992. Pharmaceutics of protein drugs. Journal of Pharmacy and Pharmacology 44:178-185.

Quadri, L.E.N., Sailer, M., Roy, K.L., Vederas, J.C. and Stiles, M.E. 1994. Chemical and genetic characterization of bacteriocins produced by *Carnobacterium piscicola* LV17B. Journal of Biological Chemistry 269:12204-12211.

Quadri, L.E.N., Sailer, M., Terebiznik, M.R., Roy, K.L., Vederas, J.C. and Stiles, M.E. 1995. Characterization of the protein conferring immunity to the antimicrobial peptide carnobacteriocin B2 and expression of carnobacteriocins B2 and BM1. Journal of Bacteriology 177:1144-1151.

Reeves, P. 1965. The bacteriocins. Bacteriological Reviews 29:24-45.

Sailer, M., Helms, G.L., Henkel, T., Niemczura, W.P., Stiles, M.E. and Vederas, J.C. 1993. ^{15}N- and ^{13}C-labeled media from *Anabaena* sp. for universal isotopic labeling of bacteriocins: NMR resonance assignments of leucocin A from *Leuconostoc gelidum* and nisin A from *Lactococcus lactis*. Biochemistry 32:310-318.

Sasaoki, K., Hiroshima, T., Kusumoto, S. and Nishi, K. 1989. Oxidation of methionine residues of recombinant human interleukin 2 in aqueous solutions. Chemical and Pharmaceutical Bulletin 37:2160-2164.

Saucier, L., Poon, A. and Stiles, M.E. 1995. Induction of bacteriocin production in *Carnobacterium piscicola* LV17. Journal of Applied Bacteriology 78:684-690.

Schillinger, U. and Holzapfel, W.H. 1990. Antibacterial activity of carnobacteria. Food Microbiology 7:305-310.

Shaw, B.G. and Harding, C.D. 1984. A numerical taxonomic study of lactic acid bacteria from vacuum-packed beef, pork, lamb and bacon. Journal of Applied Bacteriology 56:25-40.

Stoffels, G., Nissen-Meyer, J., Guomundsdottir, A., Sletten, K., Holo, H. and Nes, I.F. 1992. Purification and characterization of a new bacteriocin isolated from a *Carnobacterium* sp. Applied and Environmental Microbiology 58:1417-1422.

West, C.A. and Warner, P.J. 1988. Plantacin B, a bacteriocin produced by *Lactobacillus plantarum* NCDO 1193. FEMS Microbiology Letters 49:163-165.

Worobo, R.W., Henkel, T., Sailer, M., Roy, K.L., Vederas, J.C. and Stiles, M.E. 1994. Characteristics and genetic determinant of a hydrophobic peptide bacteriocin, carnobacteriocin A, produced by *Carnobacterium piscicola* LV17A. Microbiology (UK) 140:517-526.

Worobo, R.W., Van Belkum, M.J., Sailer, M., Roy, K.L., Vederas, J.C. and Stiles, M.E. 1995. A signal peptide secretion-dependent bacteriocin from *Carnobacterium divergens*. Journal of Bacteriology 177:3143-3149.

"Apprends des autres. Qui n'a que soi pour maître est l'élève d'un fou".

Le plus beau cadeau du monde
Og Mandino and Buddy Kaye

Chapter 3

Transcriptional Analysis and Regulation of Carnobacteriocin Production in *Carnobacterium piscicola* LV 17

A version of this paper has been accepted for publication in Gene: An International Journal on Genes and Genomes. Saucier, L., Paradkar, A.S., Frost, L.S., Jensen, S.E. and Stiles., M.E.

3.1. Introduction

Carnobacterium piscicola LV17 is a member of the group of bacteria designated as "lactic acid bacteria" (Axelsson, 1993). Bacteria included in the genus *Carnobacterium* were first designated as atypical lactobacilli (Hitchener *et al.*, 1982, Shaw and Harding, 1984). Collins *et al.* (1987) recommended new genus status for these organisms based on their inability to grow on acetate agar and at low pH, the production of L-(+)-lactic acid during glucose fermentation and on their fatty acid and peptidoglycan composition. More recently, Collins *et al.* (1991) demonstrated that a stretch of 1340 nucleotides from the 16S rRNA of *Lactobacillus maltaromicus* generated from eubacterial specific primers showed 100% sequence homology with *C. piscicola* nonetheless the authors state that further analysis must be done to confirm that these organisms are identical.

C. piscicola strains have been isolated from meat and meat products, poultry, fish and French surface-ripened soft cheeses but also from seawater (Millière *et al.*, 1994; Schillinger and Holzapfel, 1995). *C. piscicola* LV17 was first isolated from refrigerated, vacuum packaged pork (Shaw and Harding, 1984) and it was shown to be bacteriocinogenic by Ahn and Stiles (1990a). Three bacteriocins, referred to as carnobacteriocins (Cbn) A, B2 and BM1 have been purified and their respective genes have been cloned and sequenced.

The structural genes *cbnA* and *cbnB2* are carried on 72 and 61-kb plasmids named pCP49 and pCP40, respectively, whereas the structural gene for CbnBM1 is on the chromosome (Quadri *et al.*, 1994; Worobo *et al.*, 1994).

Eleven open reading frames located upstream of the CbnB2 gene (*cbnB2*) and a gene encoding an immunity protein (*cbiB2*), located downstream, were identified on a 10-kb *Pst*I fragment from pCP40. The information contained in this 10-kb *Pst*I fragment is sufficient to confer bacteriocin production in homologous and heterologous hosts. Four of these open reading frames, *cbnK*, *cbnR*, *cbnT* and *cbnD*, were found to be essential for the production of both CbnB2 and CbnBM1 and their respective immunity proteins (Quadri, 1996). CbnK and CbnR have homology with components of the histidine kinase class of sensing and signaling pathways which are ubiquitous in bacteria (Falke *et al.*, 1995), whereas CbnT and CbnD have homology with ATP-dependent translocators and accessory proteins of prokaryotic two-component, signal-sequence independent secretion systems.

By studying the growth pattern of a co-culture comprised of *Carnobacterium divergens* LV13 and *C. piscicola* LV17, we discovered that bacteriocin production by *C. piscicola* LV17 was dependent on the inoculum size. When *C. piscicola* LV17 was grown in APT broth from an initial inoculum of $\leq 10^4$ cfu/ml, bacteriocin production was not detected (Bac^-) although maximum population was reached. In contrast, when an inoculum of $\geq 10^6$ cfu/ml was used, bacteriocin production was detected in the cell-free supernatant. The Bac^- culture remained nonproducing during subsequent subculturing at high inoculation levels ($\geq 10^6$ cfu/ml), unless it was first grown on solid medium or was induced by the addition of cell-free supernatant from a carnobacteriocin-producing culture prior to the stationary phase of growth. Using purified carnobacteriocins allowed us to demonstrate that bacteriocin production is autoregulated (Chapter 2). A similar cell density dependent

phenomenon referred to as "quorum sensing" has been reported for various organisms (Fuqua *et al.*, 1994).

In this chapter, a detailed transcriptional analysis of *cbnA*, *cbnB2* and *cbnBM1* genes is presented. For simplicity, the following culture designations were used throughout this chapter: a positive control is a culture grown from a 10^7 cfu/ml inoculum of the producer strain *C. piscicola* LV17; a negative control is a culture grown from a 10^7 cfu/ml inoculum of the bacteriocin non-producer plasmidless strain, *C. piscicola* LV17C; a non-induced culture is a culture grown from a 10^4 cfu/ml bacteriocin non-producing inoculum of *C. piscicola* LV17; and an induced culture is a culture grown from a 10^4 cfu/ml inoculum of *C. piscicola* LV17 induced for bacteriocin production at time of inoculation using cell-free supernatant (1% vol./vol.) from the fully grown culture of the positive control.

3.2. Materials and methods

3.2.1. Bacterial cultures and bacteriocin assay

The strains and their growth conditions used in this study were as stated in chapter 2 (see section 2.2.1, p. 85), except that strains were routinely subcultured at 1% inoculum. For RNA isolation, cells were grown at room temperature (25°C) with agitation at 100 rpm in 500 ml of APT medium consisting of 10 g pancreatic digest of casein, 7.5 g yeast extract, 5 g NaCl, 5 g K_2HPO_4, 5 g sodium citrate, 10 g dextrose, 0.2 g Tween 80, 0.8 g $MgSO_4 \cdot 7H_2O$, 0.14 g $MnCl_2 \cdot 4H_2O$, 0.04 $FeSO_4.7H_2O$ and 1.25g Na_2CO_3 per liter, pH adjusted to 6.5 with HCl (Evans and Niven, 1951; Deibel *et al.*, 1957). Cells were harvested by centrifugation (13000 x *g*, 15 min, 4°C) when the

appropriate cell density was reached as measured by optical density at 600 nm (OD_{600}). Cell-free supernatant was obtained using a low protein binding filter (Acrodisc®, pore size 0.2 µm, Gelman Sciences, Ann Arbor, MI). Bacteriocin activity was evaluated by the spot-on-lawn assay using *Carnobacterium divergens* LV13 as the indicator strain, and expressed as arbitrary units (AU) of bacteriocin per ml (Ahn and Stiles, 1990b).

3.2.2. RNA isolation and Northern analysis

Total RNA was isolated as described by Hopwood *et al.* (1985) and RNA samples were stored in ethanol at -70°C until used. Northern analysis of *cbnA*, *cbnB2* and *cbnBM1* transcripts was performed according to the procedure described by Penfold *et al.* (1994) using 30 µg of total RNA on an 8 M urea 5% polyacrylamide gel. RNA marker III (Boehringer Mannheim Biochemica, Laval, Québec, Canada) was used as a size marker. The RNA in the gel was electroblotted for 45 min at 3 mA/cm^2 onto a Zeta probe membrane (Biorad, Hercules, CA) and RNA was UV cross-linked to the membrane using a GS Gene Linker™ (Biorad, Hercules, CA) as recommended by the manufacturer. Ten pmole of oligonucleotide probes: 5'-CCTTTTCCATAATTTACACCATC; 5'-CAATTGACCCCTGTTCGGAAAGTT; 5'-GCCCATCCACCGATAACTATTCC AGTAATAGCT corresponding to *cbnA*, *cbnB2* and *cbnBM1*, respectively, were end-labeled using 20 units of T4 polynucleotide kinase (New England Biolab, Mississauga, ON, Canada) with 30 µCi of [^{32}P]ATP (ICN Biochemical, Aurora, OH) and used for overnight hybridization at 39.6, 45.6 and 56°C for the *cbnA*, *cbnB2* and *cbnBM1* probes, respectively. The probes were synthesized according to the carnobacteriocin sequences published by Quadri *et al.* (1994) and Worobo *et al.* (1994). Hybridization solution consisted of 5X SSPE, 5X

Denhardt's solution (Sambrook *et al.*, 1989) and 5% (wt/vol.) SDS. Heat-denatured herring sperm DNA (96°C, 5 min) was used at a concentration of 200 µg/ml to eliminate nonspecific hybridization. The membrane was prehybridized for 3 h prior to addition of 1 $X10^6$ counts per minute (cpm) of labeled probe to 7 ml of hybridization solution. The membranes were washed at room temperature using a solution consisting of 0.1% SDS and decreasing concentrations of SSPE starting at 2X, until bands appeared clearly with limited background. The same Zeta probe membrane was hybridized with each of the probes after stripping with a large volume of boiling 0.1% sodium dodecyl sulfate (SDS).

3.2.3. Primer extension analysis

Primer extension analysis was performed as described by Ausubel *et al.* (1992) with the following modifications: 10 µl of 3X aqueous hybridization solution consisting of 3 M NaCl, 0.5 M Hepes pH 7.5 and 1 mM EDTA pH 8, and 6 X 10^5 cpm of end-labeled oligonucleotides was added to 15 to 30 µg of total RNA isolated from cells harvested at OD_{600} 0.5, and the volume was made up to 30 µl. Nucleic acid mixtures were heat-denatured (80°C, 5 min) prior to annealing for one hour at 46°C for *cbnA* and 56°C for *cbnB2* and *cbnBM1* as calculated by the equation for annealing temperature in Davis *et al.* (1994). Annealed nucleic acid mixtures were precipitated with ethanol. Twenty-five µl of reverse transcriptase mix [consisting of 7 µl of 2 mM dNTP, 5 µl of Avian Myeloblastosis Virus (AMV) reverse transcriptase buffer (Boehringer Mannheim Biochemica, Laval, Québec, Canada), 0.5 µl RNA Guard (Pharmacia Biotech, Baie d'Urfé, Québec, Canada) in a final volume of 25.5 µl] was added to the dry pellet. Primer extension was done with 25 units of AMV reverse transcriptase

incubated at 42°C for 1 h. Nucleic acid mixtures were then precipitated with 1 µl of 20 mg/ml glycogen (Boehringer Mannheim Biochemica, Laval, Québec, Canada), 30 µl 3 M sodium acetate, 243 µl TE pH 8 and 2X volume of ethanol. The resulting pellets were dissolved in stop solution from a Sequenase™ version 2.0 kit (USB, Cleveland, OH) and heat-denatured (80°C, 5 min) before loading onto a 8 M urea, 6% polyacrylamide gel.

3.2.4. DNA sequencing

Double-stranded DNA templates were sequenced using a Sequenase™ version 2.0 kit (USB, Cleveland, OH) and α[^{33}P]dATP (Mandel Scientific, Guelph, ON). DNA from a 2-kb *EcoR*I fragment containing the *cbnA* gene cloned into pUC118 was provided as template by R.W. Worobo. *CbnB2* template was a 16-kb *Pst*I fragment from pCP40 cloned in pCaT, isolated from *C. piscicola* LV17C2 and purified from a CsCl gradient (Sambrook *et al.*, 1989; Ahn *et al.*, 1992; Worobo *et al.*, 1994). *CbnBM1* template was isolated as described by Kraft *et al.* (1988) from *Escherichia coli* MV1193 transformed with a 4 kb *EcoR*I-*Pst*I fragment cloned in pUC118 (Quadri *et al.*, 1994). Transformants containing the *cbnB2* and *cbnBM1* template DNAs were kindly provided by L.E.N. Quadri.

3.3. Results

To understand how bacteriocin production is regulated in a culture from a high inoculum or when it is induced from a low inoculum, Northern analysis was performed on RNA isolated at different stages of growth from positive and negative controls, and from non-induced and induced cultures (Fig. 3-1). Major

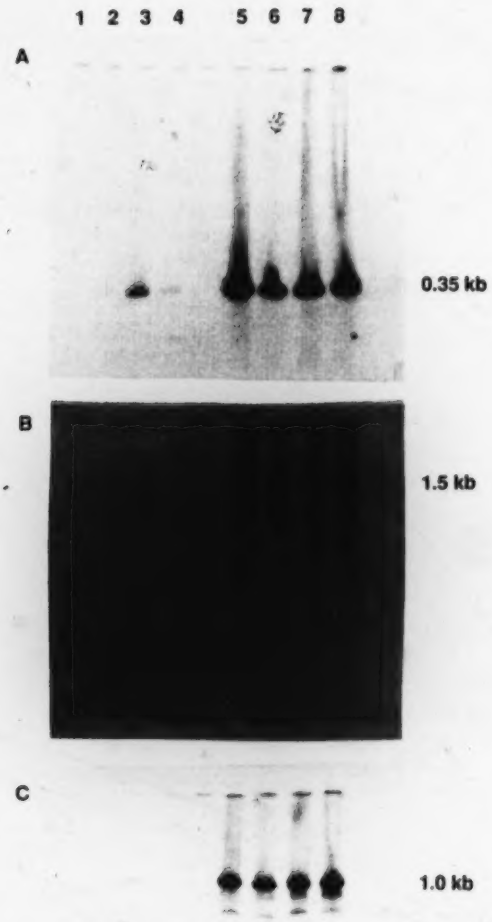


Fig. 3-1. Northern analysis of *cbnA* (**A**), *cbnB2* (**B**) and *cbnBM1* (**C**). Lane 1: negative control at OD_{600} 0.2; lanes 2-4: non-induced culture at OD_{600} 0.2, 0.5, 0.8, respectively; lane 5: positive control at OD_{600} 0.2; and lanes 6-8: induced culture at OD_{600} 0.2, 0.5, 0.8, respectively. The positive control had 200 AU/ml and induced cultures had 200, 400 and 800 AU/ml of bacteriocin activity at OD_{600} 0.2, 0.5 and 0.8, respectively.

transcripts of 0.35, 1.0 and 1.5-kb were detected for *cbnA*, *cbnBM1* and *cbnB2*, respectively, using end-labeled oligonucleotide probes synthesized according to the sequences published by Quadri *et al.* (1994) and Worobo *et al.* (1994) using RNA marker III as size markers. For *cbnB2* and *cbnBM1*, the transcript is large enough to include transcription of other genes, because the CbnB2 and CbnBM1 coding regions are only 198 bp and 183 bp, respectively. Transcripts for the three bacteriocins were not detected with the plasmidless (Bac^-) *C. piscicola* LV17C. The abundance and pattern of expression of the bacteriocin transcripts on the Northern autoradiogram (Fig. 3-1) indicated that bacteriocin production at high cell density is controlled at the transcriptional level (Fig. 3-1, compare lanes 2 and 5). When bacteriocin production was induced from a low inoculum by exogenous addition of cell-free supernatant from the fully grown culture of the bacteriocin-producing inoculum (Fig. 3-1, compare lanes 2 to 4 with lanes 6 to 8), control of bacteriocin production also appears to be controlled at the transcriptional level. With the non-induced cultures, faint bands were observed on the original autoradiogram for *cbnBM1* at OD_{600} 0.5 (not visible in Fig. 3-1) and with *cbnA* at OD_{600} 0.5 and 0.8. With induced cultures, shorter transcripts of lower intensity were detected with *cbnB2* and *cbnBM1* but not with *cbnA*.

The same membrane was used for the Northern analysis in the following order, *cbnB2, cbnBM1* and *cbnA*. Although *cbnA* was tested last, its transcript was more abundant than the other transcripts. RNA electroblotting from a SDS-PAGE gel is not quantitative and glyoxal denaturation of RNA in an agarose gel was not successful as a method for Northern analysis (data not shown). Therefore, primer extension products (oligonucleotide primers are described in Section 3.2.3) from 30 µg of RNA isolated from cells harvested at

OD_{600} 0.5 were obtained. The extension products were analyzed quantitatively from an 8 M urea, 6% PAGE gel (Free and Dorman, 1995) using ImageQuant™ and a Molecular Dynamics PhosphorImager 445SI (Molecular Dynamics Inc, Sunnyvale, CA). Results shown in Fig. 3-2 indicate that the average intensity was similar for *cbnB2* and *cbnBM1*, but 10-fold less intense than for *cbnA*. This suggests that *cbnA* is transcribed at a higher level unless *cbnB2* and *cbnBM1* transcripts are subject to a faster turnover than the *cbnA* transcript at OD_{600} 0.5.

Transcription start sites for *cbnA*, *cbnB2* and *cbnBM1* were identified by primer extension analysis. Each of the three bacteriocins are transcribed from promoters located immediately upstream of the structural genes. In the case of *cbnB2* and *cbnBM1*, their downstream genes consisting of 333 and 264-bp, respectively, are apparently cotranscribed with the bacteriocin encoding genes because the transcripts are large enough to include both genes; for *cbnB2* (198-bp) the transcript (1.5-kb) is large enough to include the *orf-β3* and could stop at the postulated inverted repeat described by Quadri *et al.* (1994).

Primer extension analysis confirmed that bacteriocin production at high cell density is controlled at the transcriptional level (Fig. 3-3, compare lanes 2 and 3), because primer extension products only appear at high level with the induced culture and the positive control. Results also suggested that autoregulation of bacteriocin is controlled at the transcriptional level (Fig. 3-3, compare lanes 2 and 3, and lanes 2 and 4, respectively). Induction of bacteriocin production by a 1% cell-free supernatant from the fully grown culture of the positive control occurs from the same promoter as was seen for the positive control (Fig. 3-3A, B and C, compare lanes 3 and 4).

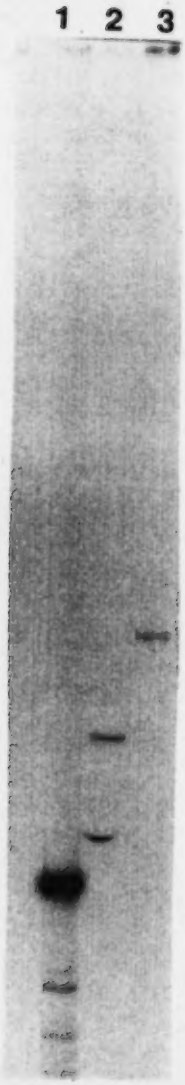


Fig. 3-2. Transcript abundance of *cbnA*, *cbnB2* and *cbnBM1* in *C. piscicola* LV17. Primer extension analysis of total RNA (30 µg) on an 8 M urea, 6% polyacrylamide gel. RNA was isolated from the positive control harvested at OD_{600} 0.5 with a bacteriocin activity of 400 AU/ml. The oligonucleotide probes had specific activities of 9.6 X 10^4, 3.0 X 10^5 and 4.1 X10^5 cpm/pmol for *cbnA* (lane 1), *cbnB2* (lane 2) and *cbnBM1* (lane 3), respectively.

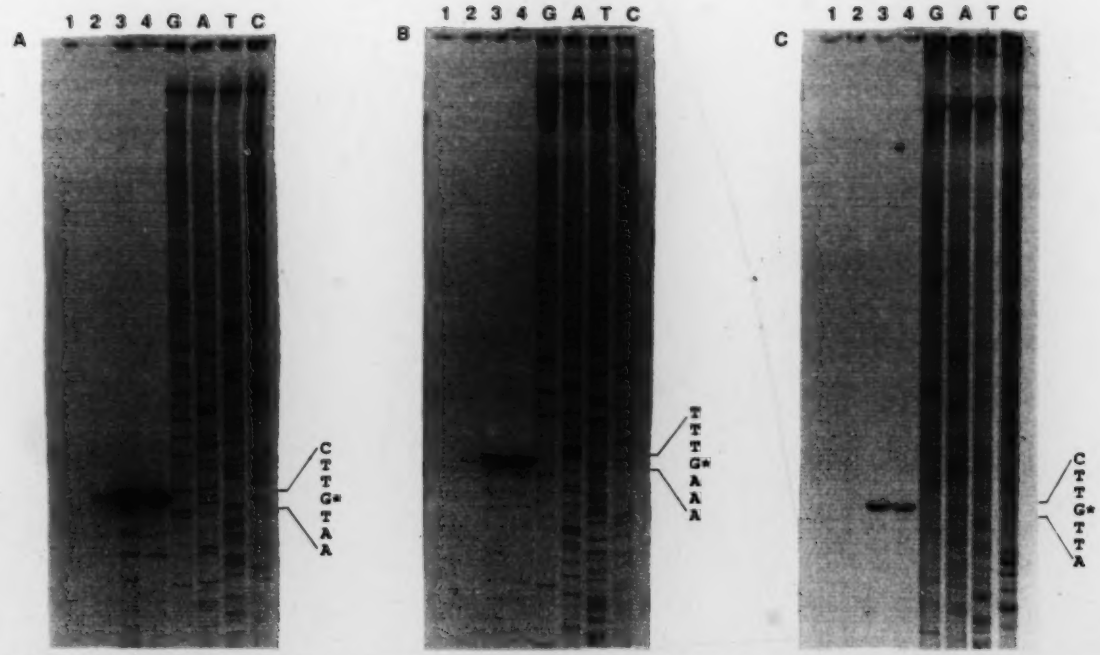


Fig. 3-3. Primer extension analysis of carnobacteriocin transcripts from *C. piscicola* LV17. Primer extension products were analyzed on an 8 M urea, 6% polyacrylamide gel using 15 µg of total RNA for *cbnA* (**A**) and, 30 µg of total RNA for *cbnB2* (**B**) and *cbnBM1* (**C**). All cultures were harvested at an OD_{600} of 0.5. The positive control and the induced cultures had bacteriocin activity of 400 AU/ml. Lane 1: negative control; lane 2: non-induced culture; lane 3: positive control; lane 4: induced culture. The nucleotides marked with an asterisk are the transcription start sites.

Promoter regions for all three bacteriocins were compared with previously reported promoters of lactic acid bacteria and *E. coli* (Table 3-1). The *cbnA* promoter differs by only one nucleotide from P59 which is the strong promoter used in pGKV259, an expression vector for lactic acid bacteria (Van der Vossen *et al.*, 1987). The promoters for *cbnB2* and *cbnBM1* are similar to a weaker promoter P21 (Van der Vossen *et al.*, 1987). Spacing between the putative -35 and -10 hexanucleotides and between the putative -10 hexanucleotides and the transcription start site for the three carnobacteriocins (Table 3-1 and Fig. 3-4) agrees with the published data for *Lactococcus* (Van de Guchte *et al.*, 1989). The presence of T residues at the -1 position is also in agreement with the data observed with *Lactococcus* (Fig. 3-4). However, the guanine at the transcription start site is not in accord with the normally prevalent A residue at that position (Van de Guchte *et al.*, 1989).

The published sequences for *cbnA*, *cbnB2* and *cbnBM1* (Quadri *et al.*, 1994; Worobo *et al.*, 1994) were aligned using ClustalW Multiple Sequence Alignment (Thompson *et al.*, 1994). The transcription start site regions of the three bacteriocins share interesting characteristics. The transcription start sites are G residues in all three cases and they are located, relative to the translation start site, at position -35 bp, for *cbnA* and *cbnB2*, and at -32 bp for *cbnBM1*, respectively (Fig. 3-3). Those particular G residues are part of a consensus sequence spanning 14 to 15 nucleotides (Fig. 3-4) which may be involved in regulation of transcription. The homology with the *sakP* and *plnA* genes is discussed below.

Table 3-1. Comparison of *cbnA*, *cbn*B2 and *cbn*BM1 promoter sequences with other promoters from lactic acid bacteria and the *E. coli* consensus sequence

Promoter	Sequence [a]	Reference
	-35 -10	
E. coli	TTGACA-14bp-tg-N-TAtAaT	Van der Vossen *et al.*, 1987
P*cbnA* [b]	TTGAC**T**-16bp-TG-a-TAGAAT	This paper
P59 [bc]	TTGAC**A**-14bp-TG-a-TAGAAT	Van der Vossen *et al.*, 1987
P*cbnB2* [b]	TT**T**C**A**-16bp-TG-a-TA**TA**GT	This paper
P*cbnBM1* [b]	TT**AT**CA-16bp-TG-c-TA**CA**GT	This paper
P21 [bc]	TTG**A**CA-15bp-TG-g-TA**TTA**T	Van der Vossen *et al.*, 1987
P23 [c]	ATGACA-14bp-TG-a-TAAAAT	Van der Vossen *et al.*, 1987
P32 [c]	TAGAAA-14bp-TG-c-TATACT	Van der Vossen *et al.*, 1987
P44 [c]	TTGTTT-15bp-ta-a-AATAAT	Van der Vossen *et al.*, 1987
lcnMa	TTGTTA-13bp-tt-a-TATAAT	Van Belkum*et al.*, 1991
lcnA	TTGTTA-13bp-tt-a-TATAAT	Van Belkum *et al.*, 1991
nisA	CTGATT-17bp-ag-a-TACAAT	Buchman *et al.*, 1988 Van de Guchte *et al.*, 1989

[a] Lower-case letters show nucleotides that are less highly conserved. N = G, A, T or C. Regions at -35 and -10, and conserved TG in front of the -10 region for lactic promoters are underlined.

[b] Differences between P59 and PCbnA, and between P21 and, PCbnB2 and PCbnBM1 are shown in bold type.

[c] *Lactococcus lactis* spp. *cremoris* Wg2 promoters (Van Belkum *et al.*, 1991)

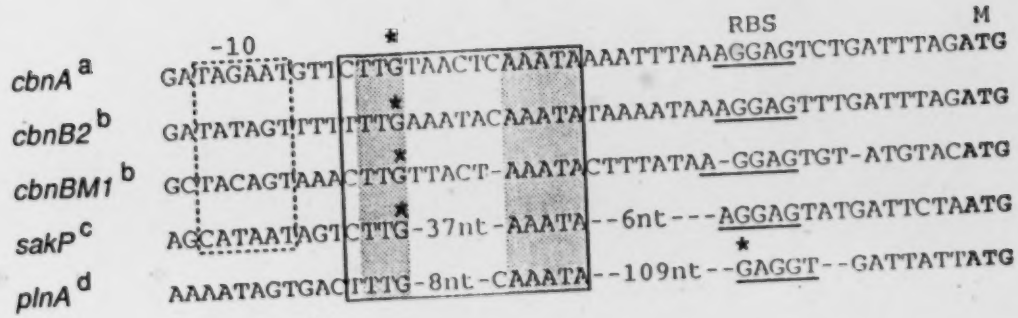


Fig. 3-4. Comparison of transcription start site regions of *cbnA, cbnB2, cbnBM1, sakP* and *plnA*. The dashed box represents the -10 region of the three carnobacteriocin promoters and the *sakP* promoter. The solid box represents the consensus sequence for the five bacteriocins; shaded nucleotides are identical for all of them. * represents the transcription start sites as determined by primer extension (Fig. 3-3; Tichaczek *et al.*, 1994; Diep *et al.*, 1996). Underlined nucleotides represent the putative ribosome binding site (RBS) and the bold nucleotides represent the translational start sites (M, methionine). The three carnobacteriocins were aligned using ClustalW Multiple Sequence Alignment, and - represents the gaps created by the program (Thompson *et al.*, 1994). Because *sakP* and *plnA* are also part of a inoculum size dependent bacteriocin production system, their genes were manually aligned to the carnobacteriocin genes. [a] Worobo *et al.*, (1994), [b] Quadri *et al.*, (1994), [c] Tichaczek *et al.*, 1994, [d] Diep *et al.*, (1996).

3.4. Discussion

Although bacteria are unicellular organisms, they often function, interact and communicate as a "community" for achieving a common goal. Examples of cell-cell interactions include: bacterial mating (e.g., pheromone production by certain enterococci); developmental interactions (e.g., signals during multicellular development of *Myxococcus*); interactions during colonization using quorum sensing (e.g., colonization of the light organ of certain fish and squid by *Phosphobacterium fischeri*; and legume nodulation by *Rhizobium*); predator-prey interaction (e.g., *Bdellovibrio* as a parasite of the periplasmic space of host cells) (Dworkin, 1991; Kaiser and Losick, 1993). In *C. piscicola* LV17, bacteriocin production in liquid medium is dependent on the level of inoculation. Our previous results demonstrated that extracellular bacteriocins are able to induce production from a low inoculum and therefore autoregulate their production. Induction must occur prior to the stationary phase of growth, otherwise bacteriocins are not detected in the supernatant (Chapter 2). Evidence for autoregulation has subsequently been published for nisin A, and evidence for bacteriocin production at high cell density in *Lactobacillus plantarum* C11 and in *Lactobacillus sake* has been reported (Diep *et al.*, 1995; Kuipers *et al.*, 1995; Eijsink *et al.*, 1996).

Northern analysis (Fig. 3-1) showed that transcription of the carnobacteriocin genes is greater in the positive control (10^7 cfu/ml culture; lane 5) compared with the non-induced culture (10^4 cfu/ml culture; lanes 2 to 4) indicating that bacteriocin production from a high inoculum is controlled at the level of transcription. The results in Fig. 3-1 also suggested that bacteriocin autoregulation is controlled at the transcriptional level because transcription is

greater in the induced culture (lanes 6 to 8) compared with the non-induced culture (lanes 2 to 4). The primer extension analyses (Fig. 3-3) also support these results. Transcription of the three carnobacteriocin genes is activated to a higher level in the positive control (10^7 cfu/ml; lane 3) and in the induced culture (lane 4) compared with the non-induced culture (10^4 cfu/ml; lane 2). Transcription was initiated from the same promoter for the three bacteriocins in the positive control (lane 3) and in the induced culture (lane 4).

The pattern of transcription confirmed the previous observation that bacteriocins must be present at inducing levels prior to the stationary phase of growth for detectable amounts of bacteriocin to be produced (Chapter 2). The weak bands in non-induced cultures (Fig. 3-1) indicate that a weak constitutive production of bacteriocin occurs and that at an inoculum of $\geq 10^6$ cfu/ml, the basal level of expression is sufficient to reach the necessary threshold level of bacteriocin production to induce full production (Fig. 3-1, lane 5). At low inoculum ($\leq 10^4$ cfu/g), the threshold level of bacteriocin production is not reached before the stationary phase and transcription is not fully activated as indicated by a decrease of transcription in lane 4 at OD_{600} 0.8 compared with lane 3 at OD_{600} 0.5 (Fig. 3-1, panels A and C). Despite the low level of transcription shown in lane 3 and 4 in panel A and C, bacteriocin production remains undetectable using the spot-on-lawn assay indicating that the bacteriocin concentration is < 50 AU/ml and that the bioassay used is not sensitive enough to detect bacteriocin production from such a low level of transcription. At this time it is not known if the reduction of transcription in stationary phase of growth is due to competition for the RNA polymerase core enzyme between specific sigma factors produced during stationary phase with

the sigma factor(s) used during exponential phase to transcribe the carnobacteriocin genes. No repressors have been identified thus far.

Determining transcript abundance using phosphorimaging analysis of primer extension products suggests that the promoter for *cbnA* is stronger compared with the promoters for *cbnB2* and *cbnBM1* (Fig. 3-2 and Table 3-1). Furthermore, comparison of the carnobacteriocin promoters with other known lactic promoters indicates that the *cbnA* promoter is stronger than the promoters for *cbnB2* and *cbnBM1* because P59 is stronger than P21 according to the results of Van der Vossen *et al.* (1987; Table 3-1). This variation of the promoter strength could explain why the cell-free supernatant of *C. piscicola* LV17A (CbnA producer) has higher activity than *C. piscicola* LV17B (producer of CbnB2 and CbnBM1) against *C. divergens* LV13 (Chapter 2). However, RNA turnover could also be implicated in the lower transcript abundance observed with *cbnB2* and *cbnBM1* compared with *cbnA*. With the induced cultures (Fig. 3-1), shorter transcripts were detected at low level with *cbnB2* and *cbnBM1* but not with *cbnA*. The shorter *cbnB2* and *cbnBM1* transcripts could be the result of a faster RNA turnover for those two transcripts compared with *cbnA*, a greater sensitivity of the RNA to in vitro handling or an indication that processing is necessary prior to translation. RNA turnover has been implicated in controlling gene expression (King and Schlessinger, 1987; Belasco, 1995) and could be at the origin of the lower inhibitory activity against *C. divergens* LV13 due to a lower expression of CbnB2 and CbnBM1. The shorter transcripts could also originate from transcription at different promoters, but primer extension analysis using total cellular RNA isolated from cells harvested at OD_{600} 0.5 did not reveal more than one promoter immediately upstream of the bacteriocin structural genes (Figs. 3-2 and 3-3). Evaluation of the half-life of

the transcripts would fully address the role of mRNA decay in bacteriocin gene expression. Despite the presence of the *cbnBM1* structural and putative immunity genes on the chromosome, no transcript was detected with the plasmidless (Bac^-) control (Fig. 3-1C). This confirmed the results obtained by Quadri (1996) that CbnBM1 production in *C. piscicola* LV17 depends on the presence of pCP40.

Multiple bacteriocin production by a single organism has been an important research goal in our laboratory. The fusion of either leader sequences or signal sequence from bacteriocins produced by lactic acid bacteria to different bacteriocins has been successful and bacteriocin production in heterologous hosts relative to the original bacteriocin producer was observed (McCormick and Van Belkum, 1996). The results presented in Fig. 3-1 showed that all three bacteriocins can naturally be coordinately transcribed during growth of the wild type strain supporting the feasibility of multiple bacteriocin production by the same organism.

The similarity of the sequence in the transcription start site region for the three carnobacteriocins described in Fig. 3-4 suggests that this particular sequence is responsible for bacteriocin autoregulation and bacteriocin production at low inoculum. Interestingly, a DNA consensus sequence similar to the one described for the carnobacteriocins was also found upstream of the structural gene in the promoter region of sakacin P (Tichaczek *et al.*, 1994) and further upstream of the promoter region of plantaricin A (Diep *et al.*, 1994, 1996). The transcription start site for sakacin P is a G residue (-65 bp relative to the translation start site) which fits into the first shaded consensus sequence presented in Fig. 3-4. Transcription start site of the inducer of bacteriocin

production in *L. plantarum* C11, plantaricin A, does not fit this consensus sequence but rather a region further upstream of the promoter region but still in a non-coding region as described in Diep *et al.* (1996); the bacteriocin structural gene(s) for *L. plantarum* C11 has yet to be confirmed. The distance that separates the 2 highly conserved sequence blocks shown in Fig. 3-4 (shaded nucleotides) and the distance that separates the 14 to 15 nucleotide sequence (solid box) from the putative ribosome binding sequence in the case of plantaricin A and sakacin P are different from the corresponding regions for the carnobacteriocins. Diep *et al.* (1996) identified another putative regulatory region consisting of 2 direct repeats of 10 nucleotides separated by 12 nucleotides upstream of the -35 regions for several genes involved in bacteriocin synthesis including *cbnA*, *cbnB2* and *cbnBM1*. The alignment obtained using ClustalW Multiple Sequence Alignment revealed the first repeat referred to as the left (L) repeat but failed to identify the second one, the right repeat. However, differences in regulation between the carnobacteriocin system with the plantaricin A and sakacin P systems might also be expected because recovery of bacteriocin production after growth on solid medium does not readily occur with plantaricin A and sakacin P (Diep *et al.*, 1995; Eijsink *et al.*, 1996) whereas it is readily recovered for the carnobacteriocins produced by *C. piscicola* LV17 (Saucier *et al.*, 1995). These consensus sequences might be a DNA binding site for a regulatory protein which could either activate under inducing conditions or repress under non-inducing conditions by affecting the processing of the RNA polymerase. The location of the described consensus sequence (Fig. 3-4) in the transcription start site region (-4 to 12), is not typical of the location of such regulatory elements in other species. In *Escherichia coli*, Busby and Ebright (1994) indicated that the DNA sequences involved in promoter activation are upstream or overlapping the -35 region for σ^{70}, which is

further upstream compared with the position of the consensus sequence found which is -4 to 12. The role of this consensus sequence in the regulation of bacteriocin production and the involvement of either activator or repressor proteins remains to be studied. For example, in the case of colicin V, a small antimicrobial peptide belonging to the class II bacteriocins synthesized by various strains of *E. coli*, bacteriocin production is iron regulated via the Fur repressor and production increases in iron limiting conditions (Chehade and Braun, 1988).

These results confirmed that bacteriocin production is not a constitutive trait in *C. piscicola* LV17 but rather that it is regulated to allow expression and production of active bacteriocin under specific conditions. The organism needs to be inoculated at $\geq 10^6$ cfu/ml for bacteriocins to be produced in liquid medium. Recovery of bacteriocin production in a non-producing culture (10^4 cfu/ml) by growth on solid medium suggests that bacteriocin induction in *C. piscicola* LV17 is characteristic of growth on a surface, for example on the surface of meat from which it was isolated, as opposed to living as free cells. A similar observation was reported for *Photobacterium fischeri* (originally named *Vibrio fischeri*), for which bioluminescence is characteristic of growth at high cell density in the light organ of certain fish and squid but not as a free living cell in sea water (Dunlup and Greenberg, 1991). In the bioluminescence regulon, the product of the *luxR* gene acts as a cell density-dependent trancriptional activator by increasing the transcription of the *luxICDABEG* operon thus allowing the expression of the necessary gene for bioluminescence. Although no direct biochemical evidence has been provided, it is believed that the transcriptional activator needs to bind to the cell density signal molecule, the product resulting from the activity of the LuxI

protein (autoinducer synthase), to activate transcription creating a positive feedback autoregulation on the *luxI* gene (Ruby and McFall-Ngai, 1992; Fuqua *et al.*, 1994; Salmond *et al.*, 1995). In *C. piscicola* LV17, bacteriocin production at high cell density is also controlled at the transcriptional level (Fig. 3-1). However, carnobacteriocins can autoregulate their production but they are not the molecules responsible for "quorum sensing" because bacteriocin production does not occur with a $\leq 10^4$ cfu/ml inoculum even after reaching a similar maximum population. It appears that bacteriocin synthesis is rather under the control of the "quorum sensing" mechanism, in which synthesis is abolished if it has not been induced before the stationary phase of growth.

Understanding of the genetic organization for production and regulation of these carnobacteriocins is essential for adequate and sound applications of *C. piscicola* LV17 as a protective culture in food. The next chapter will address the ability of *C. piscicola* LV17 to produce bacteriocins on the surface of meat inoculated at low concentration.

3.5. References

Ahn, C. and Stiles, M.E. 1990a. Antibacterial activity of lactic acid bacteria isolated from vacuum-packaged meats. Journal of Applied Bacteriology 69:302-310.

Ahn, C. and Stiles, M.E. 1990b. Plasmid-associated bacteriocin production by a strain of *Carnobacterium piscicola* from meat. Applied and Environmental Microbiology 56:2503-2510.

Ahn, C., Collins-Thompson, D., Duncan, C. and Stiles, M.E. 1992 Mobilization and location of the genetic determinant of chloramphenicol resistance from *Lactobacillus plantarum* caTC2R. Plasmid 27:169-176.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. 1992. Current protocols in molecular biology. Harvard Medical School, Boston, MA.

Axelsson, L.T. 1993. Lactic acid bacteria: classification and physiology. In: Salminen, S. and von Wright, A. (Ed.), Lactic Acid Bacteria. Marcel Dekker, Inc., New York, NY.

Belasco, J.G. 1995. mRNA degradation in prokaryotic cells: an overview. In: Belasco, J.G. and Brawerman, G. (Ed.), Control of Messenger RNA Stability. Academic Press Inc., San Diego, CA, pp. 3-12.

Buchman, G.W., Banerjee, S. and Hansen, J.N. 1988. Structure, expression, and evolution of a gene encoding the precursor of nisin, a small protein antibiotic. The Journal of Biological Chemistry 263:16260-16266.

Busby, S. and Ebright, R.H. 1994. Promoter structure, promoter recognition, and transcription activation in prokaryotes. Cell 79:743-746.

Chehade, H. and Braun, V. 1988. Iron-regulated synthesis and uptake of colicin V. FEMS Microbiology Letters 52:177-182.

Collins, M.D., Farrow, J.A.E., Phillips, B.A., Ferusu, S. and Jones, D. 1987. Classification of *Lactobacillus divergens*, *Lactobacillus piscicola*, and some catalase-negative, asporogenous, rod-shaped bacteria from poultry in a new genus, *Carnobacterium*. International Journal of Systematic Bacteriology 37:310-316.

Collins, M.D., Rodrigues, U., Ash, C., Aguirre, M., Farrow, J.A.E., Martinez-Murcia, A., Phillips, B.A., Williams, A.M. and Wallbanks, S. 1991. Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. FEMS Microbiology Letters 77:5-12.

Davis, L.G., Kuehl, W.M. and Battey, J.F. 1994. Primer extension of synthetic oligonucleotides to map 5' termini of mRNAs. In: Basic methods in molecular biology, 2nd ed. Appleton and Lange, Norwalk, CT, pp. 378-384.

Deibel, R.H., Evans, J.B. and Niven, C.F. 1957. Microbiological assay for thiamin using *Lactobacillus viridescens*. Journal of Bacteriology 74:818-821.

Diep, D.B., Håvarstein, L.S. and Nes, I.F. 1995. A bacteriocin-like peptide induces bacteriocin synthesis in *Lactobacillus plantarum* C11. Molecular Microbioligy 18:631-639.

Diep, D.B., Håvarstein, L.S. and Nes, I.F. 1996. Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* C11. Journal of Bacteriology 178:4472-4483.

Diep, D.B., Håvarstein, L.S., Nissen-Meyer, J. and Nes, I.F. 1994. The gene encoding plantaricin A, a bacteriocin from *Lactobacillus plantarum* C11, is located on the same transcription unit as an *agr*-like regulatory system. Applied and Environmental Microbiology 60:160-166.

Dunlup, P.V. and Greenberg, E.P. 1991. Role of intercellular chemical communicaton in the *Vibrio fischeri* monocentrid fish symbiosis. In: Dworkin, M. (Ed.), Microbial Cell-Cell Interaction. American Society for Microbiology, Washington, DC, pp. 219-254.

Dworkin, M. 1991. Introduction. In: Dworkin, M. (Ed.), Microbial Cell-Cell Interaction. American Society for Microbiology, Washington, DC, pp. 1-6.

Eijsink, V.G.H., Brurberg, M.B., Middelhoven, P.H. and Nes, I.F. 1996. Induction of bacteriocin production in *Lactobacillus sake* by a secreted peptide. Journal of Bacteriology 178:2232-2237.

Evans, J.B. and Niven, C.F. 1951. Nutrition of the heterofermentative lactobacilli that cause greening of cured meat products. Journal of Bacteriology 62:599-603.

Falke, J.J., Blair, D.F., Silhavy, J.T. and Schmitt, R. 1995. BLAST 1995: International conference on bacterial locomotion and signal transduction. Molecular Microbiology 16:1037-1050.

Free, A. and Dorman, C.J. 1995 Coupling of *Escherichia coli hns* mRNA levels to DNA synthesis by autoregulation: implications for growth phase control. Molecular Microbiology 18:101-113.

Fuqua, W.C., Winans, S.C. and Greenberg, E.P. 1994. Quorum sensing in bacteria: LuxR-LuxI family of cell density response transcriptional regulators. Journal of Bacteriology 176:269-275.

Hitchener, B.J., Egan, A.F. and Rogers, P.J. 1982. Characteristics of lactic acid bacteria isolated from vacuum-packaged beef. Journal of Applied Bacteriology 52:31-37.

Hopwood, D.A., Bibb, M.J., Chater, K.F., Keiser, T., Bruton, C.J., Keiser, H.M., Lydiate, D.J., Smith, C.P., Ward, J.M. and Schrempf, H. 1985. Genetic manipulation of *Streptomyces*, a laboratory manual. The John Innes Foundation, Norwich, pp. 213-230.

Kaiser, D, and Losick, R. 1993. How and why bacteria talk to each other. Cell 73:873-885.

King, T.C. and Schlessinger, D. 1987. Processing of RNA transcripts. In: Neidhart, F.C. (Ed.), *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology. American Society for Microbiology, Washington, DC, pp. 703-718.

Kraft, R., Tardiff, K.S., Krauter, K.S. and Leinwand, L.A. 1988 Using mini-prep plasmid DNA for sequencing double stranded templates with sequenase. Biotechniques 6:544-547.

Kuipers, O.P., Beerthuyzen, M.M., de Ruyter, P.G.G.A., Luesink, E.J. and de Vos, W.M. 1995. Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. The Journal of Biological Chemistry 270:27299-27304.

McCormick, J.K. and van Belkum, M.J. 1996. Department of Biological Sciences and Agricultural, Food and Nutritional Science, University of Alberta, personal communication.

Millière, J.B., Michel, M., Mathieu, F. and Lefebvre, G. 1994. Presence of *Carnobacterium* spp. in French surface mould-ripened soft-cheese. Journal of Applied Bacteriology 76:264-269.

Penfold, S.S., Usher, K. and Frost, L.S. 1994. The nature of the *traK4* mutation in the F sex factor of *Escherichia coli*. Journal of Bacteriology 176:1924-1931.

Quadri, L.E.N. 1996. Biochemical and genetic characterization of antimicrobial peptides produced by *Carnobacterium piscicola* LV17B. Ph.D. thesis, University of Alberta, Edmonton, AB.

Quadri, L.E.N., Kleerebezem, M., Kuipers, O.P., de Vos, W.M., Roy, K.L., Vederas, J.C. and Stiles, M.E. Genes from *Carnobacterium piscicola* LV17B involved in bacteriocin production and immunity: evidence for global transcriptional regulation and a novel inducer peptide. Unpublished.

Quadri, L.E.N., Sailer, M., Roy, K.L., Vederas, J.C. and Stiles, M.E. 1994. Chemical and genetic characterization of bacteriocins produced by *Carnobacterium piscicola* LV17B. The Journal of Biological Chemistry 269:12204-12211.

Ruby, E.G. and McFall-Ngai, M.J. 1992. A squid that glows at night: development of an animal-bacterial mutualism. Journal of Bacteriology 174:4865-4875.

Salmond, G.P.C., Bycroft, B.W., Stewart, G.S.A.B. and Williams, P. 1995. The bacterial 'enigma': cracking the code of cell-cell communication. Molecular Microbiology 16:615-624.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989 Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Saucier, L., Poon, A. and Stiles, M.E. 1995. Induction of bacteriocin in *Carnobacterium piscicola* LV17. Journal of Applied Bacteriology 78:684-690.

Schillinger, U. and Holzapfel, W.H. 1995. The genus *Carnobacterium*. In: Wood, B.J.B. and Holzapfel, W.H. (Ed.), The genera of lactic acid bacteria. Blackie Academic and Professional, Glasgow, pp. 307-326.

Shaw, B.G. and Harding, C.D. 1984. A numerical taxonomic study of lactic acid bacteria from vacuum-packed beef, pork, lamb and bacon. Journal of Applied Bacteriology 56:25-40.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22:4673-4680.

Tichaczek, P.S., Vogel, R.F. and Hammes, W.P. 1994. Cloning and sequencing of *sakP* encoding sakacin P, the bacteriocin produced by *Lactobacillus sake* LTH 673. Microbiology (UK) 140:361-367.

Van Belkum, M.J., Hayema, B.J., Jeeninga, R.E., Kok, J. and Venema, G. 1991. Organization and nucleotide sequences of two lactococcal bacteriocin operons. Applied and Environmental Microbiology 57:492-498.

Van de Guchte, M., Van der Vossen, J.M.B.M., Kok, J. and Venema, G. 1989. Construction of a lactococcal expression vector: expression of hen egg white lysozyme in *Lactococcus lactis* subsp. *lactis*. Applied and Environmental Microbiology 55:224-228.

Van der Vossen, J.M.B.M., Van der Lelie, D. and Venema, G. 1987. Isolation and characterization of *Streptococcus cremoris* Wg2-specific promoters. Applied and Environmental Microbiology 53:2452-2457.

Worobo, R.W., Henkel, T., Sailer, M., Roy, K.L., Vederas, J.C. and Stiles, M.E. 1994. Characteristics and genetic determinant of a hydrophobic peptide bacteriocin, carnobacteriocin A produced by *Carnobacterium piscicola* LV17A. Microbiology (UK) 140:517-526.

"What sex is to inter-personal relationships, eating is to the Human-Environment relationship, a daily consummation of our defacto marriage to the living biosphere".

Food, Sex & Salmonella
David Waltner-Toews, dvm

Chapter 4

Bacteriocin Production in *Carnobacterium piscicola* LV17 is a Characteristic of Growth at High Cell Density and on Solid Surfaces, Including Meat.

4.1. Introduction

Consumer demand plays a major role in changes in the food supply. Today, consumers demand foods that are "natural", without additives but they should remain safe and convenient for use (Rhodehamel, 1992). The search for "natural" antimicrobial compounds has led food scientists to study the inhibitory compounds produced by lactic acid bacteria (LAB). LAB have been used since ancient times for food preservation by fermentation and they produce several antimicrobial substances (Table 1-1, p. 7) including bacteriocins. Bacteriocins are proteins or protein complexes that are antagonistic to organisms closely related to the producer strain. Nisin is a class I bacteriocin (lantibiotic; Section 1.5.1, p. 25) produced by several strains of *Lactococcus lactis* ssp. *lactis*. It is commercially available and it has been used as a food preservative in more than 40 countries (Delves-Broughton, 1990). In 1988, nisin was granted GRAS status (Generally Recognized As Safe) in the United States (Fields, 1996). Bacteriocins have been the focus of intensive research for over two decades. New bacteriocins have been described and several reviews have been published (Tagg *et al.*, 1976; Klaenhammer, 1988, 1993; Jack *et al.*, 1995; Nes *et al.*, 1996).

Methods of screening for bacteriocin-producing organisms are based on inhibiting target organisms with the cell-free supernatant of the producer organism (spot-on lawn assay) or by growing cells of the producer strains on agar (direct and deferred inhibition). In the spot-on-lawn assay, serial dilutions of the cell-free supernatant to be tested are applied on an agar overlay containing the target organism. With direct and differed inhibition, the organism to be tested is inoculated onto the surface of an agar plate and overlayed with

the target organism immediately (direct) or after a specific incubation period (deferred; Hoover and Harlander, 1993; Daeschel, 1992). Depending on the method used to identify bacteriocin producers, variations in bacteriocinogenicity were reported for bacteriocin production by *Lactobacillus* species (De Klerk and Coetzee, 1961; De Klerk, 1967). The incidence of bacteriocin production in *Lactobacillus fermenti* was higher when the cells were grown on a solid medium (15.5%) compared with liquid medium (2.4%). Geis *et al.* (1983) observed a similar phenomenon with strains of *Lactococcus*. Of 280 strains tested, 23% were antagonistic to target organisms on solid medium compared with only 6% in liquid medium. Lactacin B produced by *Lactobacillus acidophilus* (Barefoot and Klaenhammer, 1983) and plantacin B produced by *Lactobacillus plantarum* (West and Warner, 1988) were only produced on solid media. The activity spectrum of the bacteriocin produced by *Lactococcus brevis* B26 varied with the method used to test for antimicrobial activity (Rammelsberg and Radler, 1990). In this case, 6 of the 9 indicator strains were inhibited by growing the bacteriocin-producing strains on agar plates whereas only one strain was inhibited using the culture supernatant in a well diffusion assay (Rammelsberg and Radler, 1990). The nature of the inhibitory compounds produced by *Lactococcus brevis* B26 was not investigated any further and no explanation was given for this change in activity spectrum.

Previously, we demonstrated that bacteriocin production by *Carnobacterium piscicola* LV17 depends on the inoculation level (Saucier *et al.*, 1995). Subsequently, similar results were reported for bacteriocin production by *Lactobacillus plantarum* C11 (Diep *et al.*, 1995) and for sakacin P produced by *Lactobacillus sake* (Eijsink *et al.*, 1996). With an inoculum of ≥

10^6 cfu/ml, bacteriocin was detected in the culture supernatant of *C. piscicola* LV17; however, with an inoculum of $\leq 10^4$ cfu/ml, bacteriocin was not detected. This bacteriocin-negative (Bac^-) culture did not produce bacteriocin even at high inoculum ($\geq 10^6$ cfu/ml) unless the organism was first grown on solid medium, or if the cell-free supernatant of a carnobacteriocin producer strain (Bac^+) was added prior to the stationary phase of growth (Chapter 2). On the basis of these results, as well as the results of transcript analysis of all three carnobacteriocins produced by *C. piscicola* LV17 (Chapter 3), we postulated that carnobacteriocins and their sulfoxide derivatives positively autoregulate production of bacteriocin. Our hypothesis states that recovery of bacteriocin production by a non-producing culture was due to cells being exposed to higher concentrations of bacteriocin when grown on a solid medium, because bacteriocin diffusion would be slower on solid than in liquid medium. This chapter provides evidence in support of this hypothesis and indicates that bacteriocin production is induced under conditions similar to growth on agar surfaces such as the surface of meat.

4.2. Materials and methods

4.2.1. Bacterial cultures and media

The strains used in this study are listed in Table 2-1 (p. 87), and their growth conditions are described in Section 3.2.1 (p. 111). After growth in liquid culture, cells were washed five times by successive centrifugation and resuspension in sterile 0.1% peptone water (Difco Laboratories, Detroit, MI) using the following regime: 16,000 x *g*, 15 min; 19,000 x *g*, 15 min; 26,000 x *g*, 15 min; 34,000 x *g*, 20 min; and 43,000 x *g*, 20 min.

4.2.2. Induction of bacteriocin production in liquid medium

Induction of bacteriocin production was studied by aseptically adding 1 or 5 ml of a cell suspension of 10^7 or 10^4 cfu/ml into dialysis tubing with a 1 kDa molecular weight cut-off (Cellu-Sep™ H1, 38 mm flat width; Membrane Filtration Products Inc, San Antonio, CA), 3.5 and 12 kDa molecular weight cut-off (Septra/Por®, 10 and 25 mm flat width, respectively; Spectrum, Laguna Hills, CA). Because carnobacteriocins produced by *C. piscicola* LV17 have a molecular weight of ≤ 5.2 kDa diffusion away from the cells was determined by the molecular weight cut-off of the tubing used. The same inoculum levels (10^7 or 10^4) cfu/ml were used in a final volume of 100 ml with no dialysis tubing as positive and negative controls, respectively. The dialysis tubing was prepared as described in Sambrook *et al.* (1989), filled and immersed in 100 ml of All Purpose Tween (APT) broth. All broth-containing flasks were then shaken at 100 rpm and incubated at room temperature (25°C). Solutions inside and outside of the tubing were tested for cell density at OD_{600} and bacteriocin production as described below (Section 4.2.6).

4.2.3. Meat systems

Meat particles were obtained by aseptically removing the liquid phase of Cooked Meat Medium (CMM; BBL, Becton-Dickinson Microbiology Systems, Cockeysville, MD) that was prepared as recommended by the manufacturer. Sterile 10 cm^2 disks of lean meat (longissimus dorsi muscle) and pork loin fat (on the outside of the longissimus dorsi muscle) were prepared as described by Greer and Jones (1991). Pork loins were obtained from the Lacombe Research Center (Agriculture and Agri-Food Canada, Lacombe, AB). Loins

were flamed twice with 95% ethanol, traces of ethanol were eliminated using a propane torch and meat disks were removed aseptically. The pH of the excised lean and fat tissue were measured using a flat glass combination electrode (Model 913600, Orion Research, Chicago, IL) and a digital pH meter (Model 671, Extech, Boston, MA). Five random readings were taken per loin and the mean of all pH readings for all of the loins was calculated. Cell counts were evaluated by plating serial dilutions of an homogenate consisting of a meat sample (equivalent to 1.25 g of dry CMM or one pork tissue disk) homogenized in 100 ml of 0.1% peptone water using a Stomacher (A.J. Seward, St-Edmunds, England).

4.2.4. Bacteriocin production on meat particles and on lean and fat pork tissue

Meat particles from CMM and lean and fat pork tissue were inoculated from a fully grown 24 h Bac^+ culture diluted in either CMM, APT broth or 0.85% NaCl to achieve cell concentrations ranging from 10^7 to 10^2 cfu/g or cfu/cm^2 for meat particles and pork tissue, respectively. Meats were immersed in the inoculum for either 30 min, 15 min or 15 sec in a final volume of 6 ml for meat particles (the number of particles corresponded to 1.25 g of dry CMM) and in 200 ml for pork tissue disks. In the case of pork tissue, disks of meat were allowed to drip for 15 min on a sterile grid to remove excess moisture and to allow bacteria to adhere to the meat. Bacteriocin production was assessed daily for 2 or 3 days as described below (Section 4.2.6). *Leuconostoc gelidum* UAL187 was used as a positive control because bacteriocin production was demonstrated on refrigerated vacuum packaged pork loin meat (McMullen *et al.*, 1995). *C. piscicola* LV17C (Bac^-) was used as a negative control

(Table 2-1, p. 87). All pork lean and fat disks and meat particle samples were incubated at 25°C.

4.2.5. Bacteriocin stability on meat particles and lean pork

A bacteriocin-positive, fully grown culture was centrifuged and the supernatant was sterilized using a low protein binding filter (Acrodisc®, pore size 0.2 µm, Gelman Sciences, Ann Arbor, MI). APT broth or 0.85% NaCl was used to dilute the cell-free supernatant to 50, 10, 5 and 1% of the original concentration. To determine the stability of bacteriocin on meat, meat particles and lean pork disks were immersed in diluted supernatant solutions for 15 min and bacteriocin activity was determined using the assay described below (Section 4.2.6). Samples were incubated at 25°C and analyzed daily for 2 days.

4.2.6. Assay for bacteriocin activity

To determine bacteriocin activity in liquid culture, the spot-on-lawn technique was done using heat-treated (65°C for 30 min) or filter-sterilized (Acrodisc®, pore size 0.2 µm, Gelman Sciences, Ann Arbor, MI) supernatants of the producer strains. *C. divergens* LV13 was used as the indicator strain. Results were expressed as arbitrary activity units (AU) of bacteriocin per ml (Ahn and Stiles, 1990b).

To test for bacteriocin activity on meat particles obtained from 1.25 g of dry CMM, 30 ml of soft APT agar (50°C) was added to the particles prior to heat treatment (65°C for 30 min) and the mixture was cooled to 50°C before adding

1% (vol./vol.) of the indicator strain. The agar-meat particle-indicator strain mixture was then poured into a 150 X 15 mm petri dish. A second layer of soft APT agar inoculated with 1% of the indicator strain was poured over the first layer to ensure that the meat particles were completely covered.

A similar procedure was used to study bacteriocin activity on disks of pork tissue using a 150 X 15 mm petri dish or a 400 ml beaker instead of a petri dish. Alternatively, a method based on the well diffusion assay was used (Daeschel, 1992; Hoover and Harlander, 1993). In this case, a piece of meat was placed in a sterile 50 ml polypropylene tube (Becton Dickinson Microbiology Systems, Cockeysville, MD) and it was entirely covered with soft APT agar. The meat and soft agar mixture was heated at 65°C for 30 min and poured into a well (10 cm diameter) in 60 ml of APT agar in a 400 ml beaker. After 30 min, 5 µl of Pronase E solution (10 mg/ml; Sigma, St-Louis, MO) was spotted beside the meat and allowed to dry before overlayering with soft APT agar inoculated with the indicator strain (1% vol./vol.).

Efficiency of the heat inactivation of the producer strain on meat particles and lean and fat pork tissue in soft APT agar was tested by inoculating four tubes of APT broth (1% vol./vol.) with heated agar-meat mixture which had been homogenized (2 min) in 100 ml of 0.1% peptone water using a Stomacher (A.J. Seward, St-Edmunds, England). Over a minimum of 10 days, growth of survivors was evaluated by an increase in the OD_{600} in the inoculated tube of APT broth. Because the possibility of recovering survivors is reduced by the dilution of the heated agar-meat mixture in 0.1% peptone water, efficiency of the heat inactivation of the producer strain was also tested by pouring the agar-meat mixture directly into a 150 X 15 mm petri dish (without

homogenization in 0.1% peptone water). Growth of survivors was then evaluated for a minimum of 10 days by scoring colony formation on the plates during incubation at 25°C. However, small colonies are not easily differentiated from meat debris. Plates were incubated anaerobically (10% CO_2, 90% N_2) in a Gas Pak® jar (BBL, Becton-Dickinson Microbiology Systems, Cockeysville, MD).

4.3. Results

Cells from a fully grown carnobacteriocin-producing culture, washed with 0.1% peptone water to remove all traces of supernatant, produced bacteriocin in liquid medium when the inoculum was $\geq 10^6$ cfu/ml but not at $\leq 10^5$ cfu/ml. Dialysis tubing was used in an attempt to demonstrate that if the bacteriocin is separated from the cells, bacteriocin production will not be induced in the cells inside the tubing. Even in the tubing with the largest pore size (12 kDa), which represents more than twice the molecular size of the carnobacteriocins, bacteriocin was not detected in the dialysate with any of the inoculation levels tested (Table 4-1). Bacteriocin production was detected inside the dialysis tubing originally inoculated at 10^7 or 10^4 cfu/ml. When the negative control from a 100 ml culture with no dialysis tubing reached an OD_{600} of 0.2 or 0.8, cell density and bacteriocin activity were tested on solutions inside and outside of the dialysis tubing. Bacteriocin activity was detected in the 1 ml volume of 10^4 cfu/ml culture inside the tubing at the sampling times corresponding to both OD_{600} of 0.2 and 0.8 for the negative control but from the 5 ml volume of 10^4 cfu/ml culture only when the negative control reached an OD_{600} of 0.8. Bacteriocin activity was never detected in the solution outside of the dialysis tubing at all cell concentrations and all dialysis

Table 4-1. Production of bacteriocin [a] by *C. piscicola* LV17 cells growing in dialysis tubing

Inoculum (cfu/ml)	MW cut-off (kDa)	OD_{600}		Bacteriocin production (AU/ml)	
10^7	+ control [b]	0.245		400	
10^7	+ control [b]	0.800		400	
10^4	– control [b]	0.255		neg [c]	
10^4	– control [b]	0.825		neg	
1 ml volume in 2 cm of tubing		**outside**	**inside**	**outside**	**inside**
10^4 [d]	12	0.020	ND [e]	neg	100
10^4 [d]	3.5	0.000	ND	neg	400
10^4 [d]	1	0.040	ND	neg	200
5 ml volume in 4 cm of tubing					
10^7 [f]	12	0.020	0.220	neg	400
10^4 [g]	12	0.015	0.305	neg	neg
10^4 [g]	1	0.005	0.340	neg	neg
10^4 [h]	12	0.005	> 1	neg	800
10^4 h	1	0.025	> 1	neg	800

a Data shown are the lowest levels of bacteriocin production obtained from two experimental replicates. Sensitivity of the bioassay is ± one doubling dilution.

b Controls, dialysis tubing was not used; aliquot was taken from the 100 ml culture.

c neg = negative (< 50 AU/ml).

d Sample was analyzed at the same time as the negative control at OD_{600} 0.255.

e ND = not determined.

f Sample was analyzed at the same time as the positive control at OD_{600} 0.245.

g Sample was analyzed at the same time as the negative control at OD_{600} 0.240.

h Sample was analyzed at the same time as the negative control at OD_{600} 0.825.

tubing molecular weight cut-offs tested.

Inhibition of the target strain *C. divergens* LV13 was used to assess the stability of the bacteriocin on meat particles and on lean pork. Meat particles were immersed in solutions containing decreasing concentrations of cell-free supernatant of either *C. piscicola* LV17 or *L. gelidum* UAL187 diluted in APT broth or 0.85% NaCl and results for the effect of storage on bacteriocin activity are shown in Table 4-2. For all variables tested (diluents, % supernatant, time of incubation), a similar trend in bacteriocin stability was observed for either *C. piscicola* LV17 or *L. gelidum* UAL187 when dilutions of the active supernatant were applied on meat particles. The diluent has little effect on bacteriocin activity but the bacteriocin activity decreased with decreasing percent supernatant and incubation time. These results indicate that at high concentrations (≥ 50% supernatant), bacteriocin activity on meat particles was stable for 2 days at room temperature in the absence of indigenous microflora or producer organisms.

Because heat treatment is used to inactivate the bacteriocin producing strain during assay of bacteriocin activity on meat, it was necessary to test the killing efficiency of the heat treatment. Heat treatment at 65^{o}C for 30 min was found to be sufficient to inactivate *C. piscicola* LV17 and *L. gelidum* UAL187. However, small numbers of organisms that either survived the heat treatment or contaminated the samples after heat treatment were observed. None of the contaminants produced antimicrobial substances active against the indicator strain *C. divergens* LV13 and they were either morphologically different to *C. piscicola* LV17 and *L. gelidum* UAL187 by microscopic examination or failed to grow as a dispersed culture in liquid medium.

Table 4-2. Bacteriocin activity [a] from meat particles immersed in cell-free supernatant of *C. piscicola* LV17 or *L. gelidum* UAL187

Strain	Diluent	% supernatant	Daily bacteriocin activity (days) 0	1	2
C. piscicola LV17	APT	100	+++	++	++
		50	+++	++	++
		10	++	+	-
		5	+	-	ND [b]
		1	-	ND	ND
		0	-	ND	ND
	0.85% NaCl	50	++	++	++
		10	++	+	-
		5	+	-	-
		1	-	ND	ND
		0	-	ND	ND
L. gelidum UAL187	APT	100	++	++	++
		50	++	++	++
		10	++	++	+
		5	++	-	-
		1	-	ND	ND
		0	-	ND	ND
	0.85% NaCl	50	++	++	++
		10	++	++	+
		5	++	-	-
		1	-	ND	ND
		0	-	ND	ND

[a] Bacteriocin activity was evaluated by inhibition of the indicator strain, *C. divergens* LV13. Results are expressed as follows: +++ = indicator strain is totally inhibited, the plate is all clear; ++ = clearly visible zone of inhibition; + = faint, but visible zone of inhibition; - = no inhibition of the indicator strain. Meat particles were immersed in the supernatant solutions for 15 min.

[b] ND = not determined.

Inhibition of the target strain varied between the heated and the unheated samples for both the meat particles (Fig. 4-1) and lean pork disks (Fig. 4-2) for samples immersed in cell-free supernatant of *C. piscicola* LV17 diluted with APT broth to 400 AU of bacteriocin per ml. Heat treatment at 65°C for 30 min allowed the release of the bacteriocin from the meat matrix, which influenced detection of bacteriocin activity, as demonstrated by the level of inhibition of the indicator strain (Figs. 4-1 and 4-2). The pronase spot was used to show that the zone of clearing of the indicator lawn was due to a proteinaceous compound. With the heated meat particles, the indicator strain was totally inhibited except where the pronase E was spotted; whereas, with the unheated meat particles, growth of the indicator strain was observed not only where the pronase E was spotted, but also at the edge of the plate, away from the meat particles (Fig. 4-1). On lean pork, zones of inhibition were only observed when the sample was not heated (Fig. 4-2). In this case, the bacteriocin released from the meat by the heat treatment appeared to be diluted out in the soft APT to such an extent that the threshold inhibitory concentration was not obtained and no zone of inhibition was observed. This prompted the development of another detection method for pork tissue based on the well diffusion assay (Section 4.2.6) in order to avoid dilution of the bacteriocin. A comparison of Figs. 4-1 and 4-2 also showed that the indicator strain was inhibited more efficiently with the meat particles compared with lean pork disks. Meat particles are more porous in nature and appeared to absorb more bacteriocin from the supernatant than the lean pork tissue. The difference in the inhibition of the target strain might also be due to the bacteriocin binding more tightly to lean pork compared with meat particles.

The first attempt to evaluate bacteriocin production *in situ* in meat

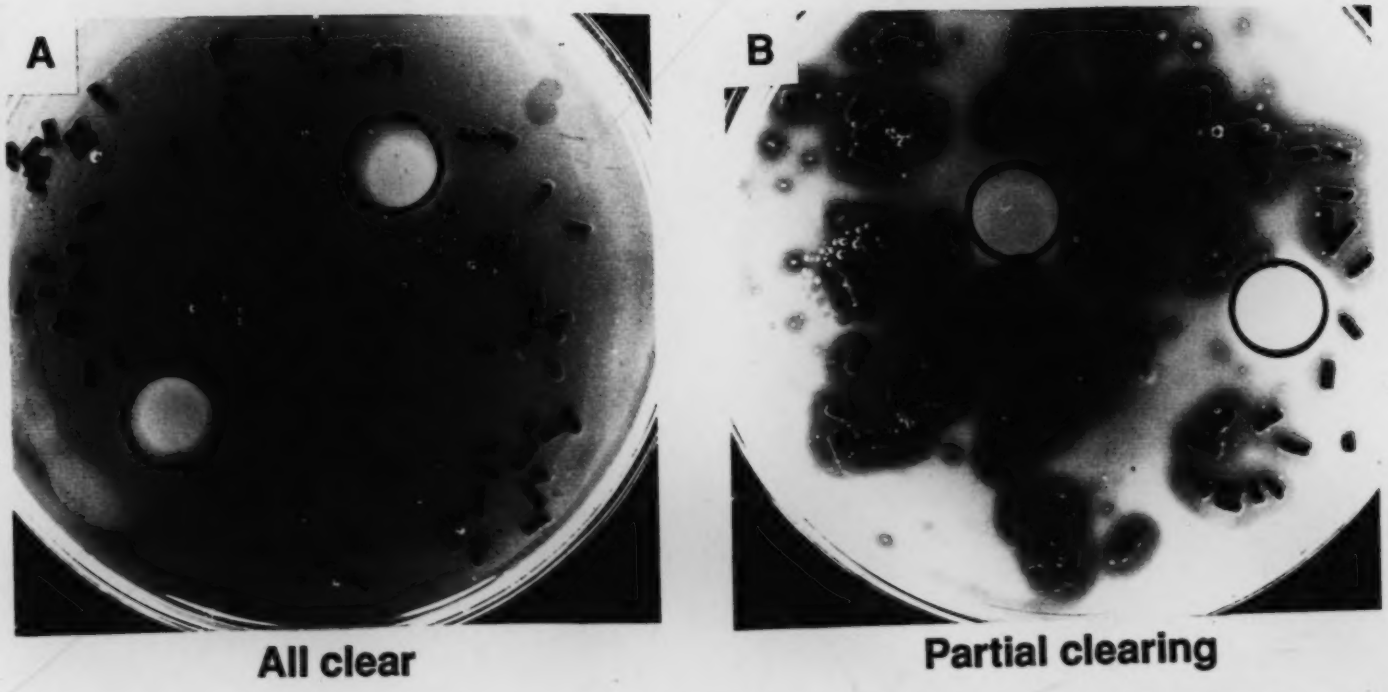


Fig. 4-1. Bacteriocin activity from meat particles immersed in cell-free supernatant of *C. piscicola* LV17. Bacteriocin activity was evaluated by inhibition of the indicator strain, *C. divergens* LV13. "All clear" means that growth of the indicator is totally inhibited except where the pronase E solution was spotted (black circles). "Partial clearing" indicates that growth of the indicator was not fully inhibited throughout the surface of the plate. Bacteriocin activity in the immersion solution remains at 400 AU/ml by the end of the 15 min immersion. Meat particles were heated (65°C, 30 min; A) or not heated (B) in 30 ml of soft APT prior to addition of the indicator strain (1% vol./vol.).

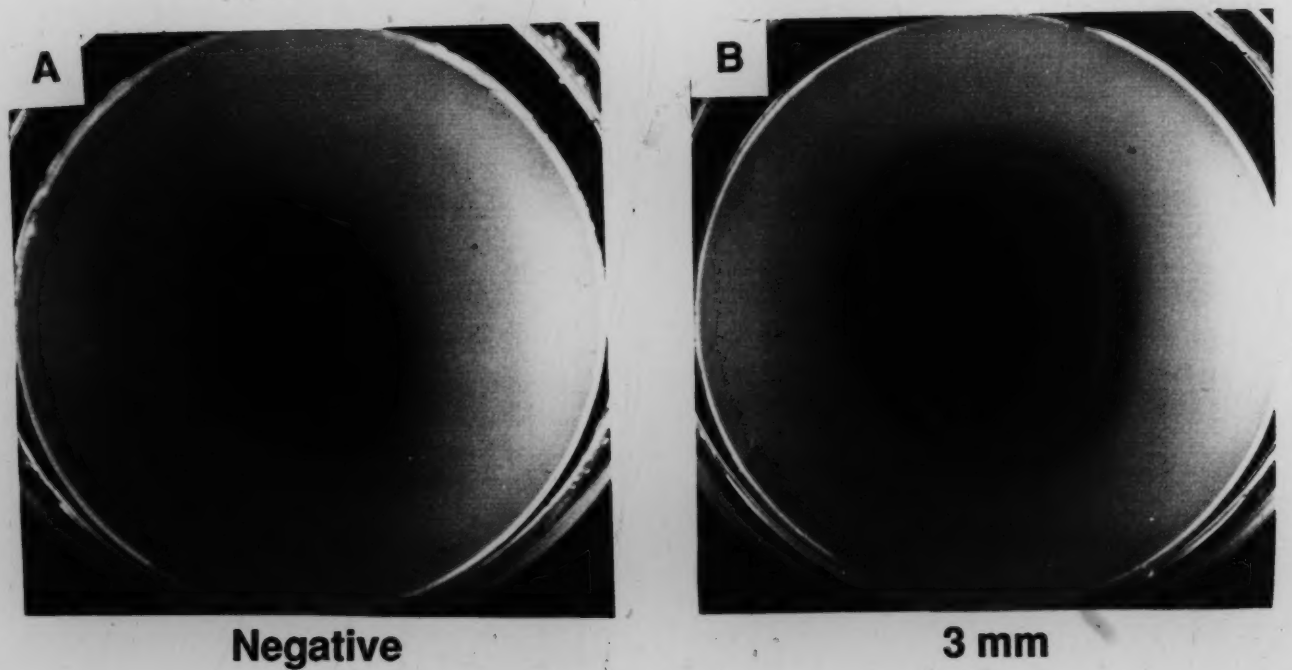


Fig. 4-2. Bacteriocin activity from lean pork meat immersed in cell-free supernatant of *C. piscicola* LV17. Immersion was as described in Fig. 4-1. Bacteriocin activity was evaluated by inhibition of the indicator strain, *C. divergens* LV13. The width of the zone of inhibition around the meat was measured in mm. Samples were heated (A) or not heated (B) in 30 ml of soft APT prior to addition of the indicator strain (1% vol./vol.).

particles was done using 0.85% NaCl as diluent and the meat particles were in contact with the inoculum for 15 sec (Table 4-3). The inoculation liquid was aseptically removed after immersion, leaving only the meat particles on the bottom of the tube for the following 2 days of incubation at 25°C. Bacteriocin activity was not detected on the meat particles inoculated with *L. gelidum* UAL187 at any of the inoculation levels tested. However, bacteriocin activity was observed with the meat particles inoculated with *C. piscicola* LV17 at 10^7 and 10^2 cfu/g but not at 10^4 cfu/g. The lack of bacteriocin production was not due to a lack of growth of the organism because both producer strains grew to maximum cell population (> 10^8 cfu/g) at each of the inoculation levels tested.

To further investigate the impact of diluent and time of immersion on bacteriocin production, CMM, APT broth and 0.85% NaCl were used and the results are presented in Table 4-4. The levels of inoculation were essentially the same for all diluents and times of immersion. *In situ* bacteriocin production varied according to the diluent used. When *C. piscicola* LV17 was diluted in APT broth, bacteriocin activity appeared slightly better than in CMM and was the lowest in 0.85% NaCl. With 0.85% NaCl, no bacteriocin activity was detected at 10^4 cfu/g. An immersion time of 30 min gave the best results in terms of bacteriocin activity when the meat particles were inoculated at 10^4 cfu/g; this immersion time was therefore used for the subsequent experiments along with APT broth as the diluent.

As stated above, bacteriocin production *in situ* by *L. gelidum* UAL187 on refrigerated vacuum packaged pork loin has been reported (McMullen et al., 1995) and is not dependent on inoculum size as was observed for *C. piscicola* LV17 (Chapter 2). *L. gelidum* UAL187 was therefore used in this study as a

Table 4-3. Bacteriocin activity [a] from meat particles inoculated with *C. piscicola* LV17 or *L. gelidum* UAL187

Producer Strains	Incubation time (days)	Bacteriocin activity at different inoculum levels (cfu/g) [b]		
		10^7	10^4	10^2
C. piscicola LV17	0	-	-	-
	1	++	-	-
	2	++	-	+
	3	++	-	+
L. gelidum UAL187	0	-	-	-
	1	-	-	-
	2	-	-	-
	3	-	-	-

a Bacteriocin activity was evaluated by inhibition of the indicator strain, *C. divergens* LV13. Results are expressed as follows: ++ = clearly visible zone of inhibition; + = faint, but visible zone of inhibition; - = no inhibition of the indicator strain. Meat particles were immersed in 0.85% NaCl solution containing the appropriate number of cells to achieve the desired inoculation levels.

b At the end of the experiment all cells reached maximum population of $\geq 10^8$ cfu/g.

Table 4-4. Effect of diluent and immersion time on bacteriocin activity [a] from meat particles inoculated with *C. piscicola* LV17 at various cell densities

Inoculum (cfu/g)	Immersion time	Incubation time (days)	Diluent tested for bacteriocin activity		
			CMM	APT	NaCl 0.85%
10^7	30 min	1	++	+++	++
	15 min		++	+++	++
	15 sec		++	+++	++
	30 min	2	++	+++	++
	15 min		++	+++	++
	15 sec		++	+++	++
10^4	30 min	1	+	+	-
	15 min		-	+	-
	15 sec		-	-	-
	30 min	2	+	++	-
	15 min		+	++	-
	15 sec		+	+	-

[a] Bacteriocin activity was evaluated by inhibition of the indicator strain, *C. divergens* LV13. Results are expressed as follows: +++ = indicator strain is totally inhibited, the plate is all clear; ++ = clearly visible zone of inhibition; + = faint, but visible zone of inhibition; - = no inhibition of the indicator strain. Bacteriocin activity was negative at time zero for all inoculation levels tested.

positive control for bacteriocin production on meat systems. Bacteriocin production in meat particles inoculated with *L. gelidum* UAL187 reached the same level of inhibition of the indicator strains regardless of the inoculation level (Table 4-5). Bacteriocin production was also detected in meat particles inoculated with *C. piscicola* LV17 at all of the inoculation levels tested; however, production was slightly lower at 10^4 cfu/g. These results show that, in contrast to the situation in liquid medium (Chapter 2), bacteriocin can be produced by *C. piscicola* LV17 *in situ* from a low inoculation level on meat particles.

Meat particles were obtained from CMM and were therefore subjected to sterilization by heat (autoclaving 121°C, 15 min). Bacteriocin production on fresh meat was also investigated using lean and fat disks from pork loins, because *C. piscicola* LV17 was originally isolated from vacuum packaged pork. The pH of the lean portion of pork was 5.61 ± 0.06 and the fat portion was 6.58 ± 0.12. *C. piscicola* LV17 produced bacteriocin on pork fat whereas *L. gelidum* UAL187 produced bacteriocin on lean meat (Table 4-6 and Fig. 4-3). However, bacteriocin production by *L. gelidum* UAL187 on lean meat was weak and delayed compared with the bacteriocin production by *C. piscicola* LV17 on fat (Table 4-6). When the meat was inoculated with a high inoculum (an undiluted fully grown culture was used to inoculate the meat at 10^7 cfu/cm^2), bacteriocin activity was detected at the time of inoculation (t = 0 day), similar to the results presented in Table 4-2. The bacteriocin activity in this case came from the inoculation solution not from the production of bacteriocin *in situ*. *C. piscicola* LV17 produced bacteriocin from a low inoculum of 3.01 log cfu/cm^2 on pork fat; bacteriocin activity was detected after 1 day of incubation at 25°C. However, bacteriocin activity was not detected at subsequent sampling times. A similar

Table 4-5. Bacteriocin activity [a] from meat particles inoculated with *C. piscicola* LV17 or *L. gelidum* at various cell densities

Time (days)	Cell count (log cfu/g)	Bac [b] activity	Cell count (log cfu/g)	Bac activity	Cell count (log cfu/g)	Bac activity
***C. piscicola* LV17**						
0	6.70	-	3.74	-	1.95	-
1	9.70	+++	9.26	-	7.20	-
2	9.76	+++	9.66	++	9.38	+++
3	9.70	+++	9.64	++	9.68	+++
***L. gelidum* UAL187**						
0	6.69	-	3.84	-	1.94	-
1	9.21	+++	8.45	-	5.68	-
2	9.18	+++	9.16	+++	9.41	+++
3	9.73	+++	9.48	+++	9.73	+++

[a] Bacteriocin activity was evaluated by inhibition of the indicator strain, *C. divergens* LV13. Results are expressed as follows: +++ = indicator strain is totally inhibited, the plate is all clear; ++ = clearly visible zone of inhibition; + = faint, but visible zone of inhibition; - = no inhibition of the indicator strain. Meat particles were immersed for 30 min in APT broth containing the appropriate number of cells to achieve the desired inoculation levels. *C. piscicola* LV17C was used as a negative control. For this strain and at t = 0 d, log cfu/g was 6.50, 3.82 and 1.85, respectively: no bacteriocin activity was detected throughout the experiment.

[b] Bac = bacteriocin.

Table 4-6. Bacteriocin activity [a] from lean and fat pork tissue inoculated with *C. piscicola* LV17 or *L. gelidum* UAL187

Time (days)	Cell count (log cfu/cm^2)	Bac [b] activity	Cell count (log cfu/cm^2)	Bac activity	Cell count (log cfu/cm^2)	Bac activity
***C. piscicola* LV17**						
Lean						
0	7.72	++	5.17	-	3.21	-
1	7.80	-	8.08	-	5.42	-
2	7.45	-	8.24	-	8.26	-
3	7.70	-	8.28	-	7.98	-
Fat						
0	7.51	++	5.06	-	3.01	-
1	8.46	-	8.61	++	7.53	++
2	ND [c]	ND	8.21	-	8.45	-
3	ND	ND	7.95	-	8.25	-
***L. gelidum* UAL187**						
Lean						
0	6.07	++	4.04	-	2.36	-
1	7.59	-	7.83	-	5.21	-
2	9.09	-	9.40	-	8.78	-
3	9.11	+	9.28	-	9.62	+
Fat						
0	ND	ND	3.84	-	1.78	-
1	ND	ND	7.88	-	6.45	-
2	ND	ND	8.93	-	8.50	-
3	ND	ND	8.40	-	8.55	-

a Bacteriocin activity was evaluated by inhibition of the indicator strain, *C. divergens* LV13. Results are expressed as follows: ++ = clearly visible zone of inhibition; + = faint, but visible zone of inhibition is observed; - = no inhibition of the indicator strain. Pork tissue disks were immersed for 30 min in APT broth containing the appropriate number of cells to achieve the desired inoculation levels. Because bacteriocin production by *L. gelidum* UAL187 is not inoculum dependent and because bacteriocin production was always detected at an inoculum ≥ 6 log cfu/ml with *C. piscicola* LV17, the limited amount of fat disks were first used for inoculation at low levels. *C. piscicola* LV17C was used as a negative control. For this strain and at t = 0 d, log cfu/cm^2 was 7.59 on lean pork tissue and 7.84 on fat pork tissue: no bacteriocin activity was detected throughout the experiment.

b Bac = bacteriocin.

c ND = not determined.

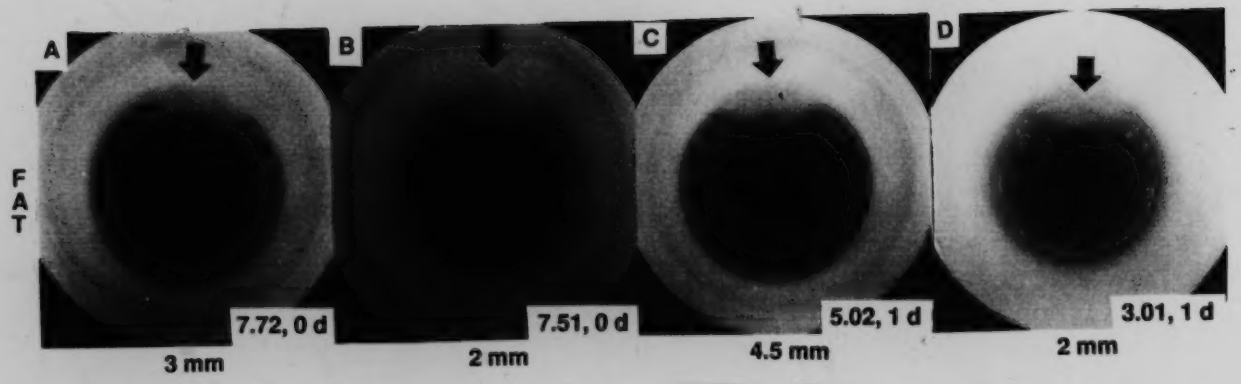


Fig. 4-3. Bacteriocin activity from pork fat tissue (A to D) inoculated with *C. piscicola* LV17 was evaluated by inhibition of the indicator strain, *C. divergens* LV13. Initial cell concentrations (log of cfu/cm^2) and incubation time are indicated in the right lower corner of each panel. The width of the zone of inhibition around the meat is measured in mm and is shown below each panel. Arrows indicate where pronase E solution was spotted.

peak of bacteriocin activity was also observed when bacteriocin was produced *in situ* from an inoculum of 5.06 log cfu/cm^2.

The dependence on inoculum size for bacteriocin production by *C. piscicola* LV17 was first observed in liquid culture incubated at 25°C and the experiments done with meat particles and pork tissues were also performed at 25°C to avoid the introduction of another variable. However, meat is normally stored at refrigeration temperature to preserve it, not at 25°C, and the effect of temperature on bacteriocin production *in situ* remains to be addressed. In fact, when liquid cultures from a low inoculum (≤ 10^4 cfu/ml) were incubated at 10 and 4°C, bacteriocin activity was detected in the fully grown culture but not in the negative control incubated at room temperature (Table 4-7). These results indicate that bacteriocin production can be induced at low temperatures in a low inoculum culture.

4.4. Discussion

Northern analysis of the bacteriocin structural genes (Chapiter 3) indicated that transcription levels vary from a basal level of expression at low inoculum (10^4 cfu/ml) to a higher level of transcription with a high inoculum (10^7 cfu/ml). Presumably, this basal level of bacteriocin production is sufficient to reach the threshold concentration of bacteriocin necessary to induce detectable levels of bacteriocin when the inoculum is ≥ 10^6 cfu/ml. Results with washed cells confirmed that the basal level of bacteriocin production was sufficient to induce bacteriocin production to an optimum level. Bacteriocin production was still detected at ≥ 10^6 cfu/ml even though the cells were washed 5 times with peptone water to remove traces of supernatant (see

Table 4-7. Effect of temperature on bacteriocin production by *C. piscicola* LV17 [a]

Temperature (°C)	Bacteriocin production (AU/ml)	time of analysis (days)
10^7 cfu/ml		
Room temperature	800	1
10	800	4
4	400	9
10^4 cfu/ml		
Room temperature	neg [b]	1.5
10	400	7
4	100	13
10^2 cfu/ml		
Room temperature	neg	2
10	200	8.5
4	neg	16

[a] Results are expressed as a mean of 3 replicates at 10^7 and 10^4 cfu/ml and, of two replicates at 10^2 cfu/ml. Cultures were sampled when cells had reached an OD_{600} of 0.80 using a culture that was vortexed daily to determine when they were fully grown.

[b] neg = negative (< 50 AU/ml).

Chapter 3).

Carnobacteriocins produced by *C. piscicola* LV17 have molecular weights that range from 4.5 to 5.2 kDa (Quadri *et al.*, 1994; Worobo *et al.*, 1994). Therefore, the dialysis tubing with 12 kDa cut-off should have allowed the bacteriocin to diffuse into the surrounding APT medium. However, several bacteriocins have been reported to form aggregates which could increase their relative molecular size (Andersson, 1986; Mørtvedt and Nes, 1989; Barefoot and Klaenhammer, 1983). In addition, if the bacteriocins can bind to the surface of the tubing, diffusion of bacteriocin would be reduced. Loss of bacteriocin activity in dialysis tubing was reported before by Rammelsberg and Radler (1990). Another possible explanation for the negative result is that the sensitivity of the method used for assaying bacteriocin might not have been sufficient to detect the bacteriocin, i.e., the concentration of bacteriocin in the dialysate was < 50 AU/ml.

When the cells from a non-producing inoculum (10^4 cfu/ml) were isolated in dialysis tubing of different molecular cut-off sizes, bacteriocin production was detected (Table 4-1). If dialysis allows a repressor molecule to be titrated out, its molecular weight must be less than a 1 kDa because bacteriocin activity was observed in dialysis tubing with 1 kDa cut-off size. An alternative explanation for bacteriocin production from the low inoculum liquid culture is that the cells interacted with the dialysis tubing and used it as a solid surface to produce an induction effect similar to that observed on solid medium. This would account for the difference between the 1 ml and the 5 ml culture. To manipulate and clamp the dialysis tubing and to avoid contamination on the outside of the tubing, a minimum length of 1.5 to 2 cm was needed. Therefore,

the ratio of the tubing surface area to culture volume was greater with the 1 ml culture compared with the 5 ml culture because 2 cm of tubing was used for the 1 ml and 4 cm was used for the 5 ml volume. Alternatively, if a small amount of bacteriocin was produced from the low inoculum, the larger volume will have increased the dilution factor delaying the attainment of the inducing threshold concentration of bacteriocin. The higher cell density of the culture inside the tubing indicates that availability of nutrients or diffusion of inhibiting end products of cellular metabolism promoted more extensive growth and allowed the cells from a low inoculum to produce enough bacteriocin to reach the inducing threshold.

Carnobacteriocins produced by *C. piscicola* LV17 are stable when heated at 65°C for 30 min under the conditions described by Quadri *et al.* (1994) and Worobo *et al.* (1994). The results presented in Figs. 4-1 and 4-2 indicate that heat treatment helped to release the bacteriocin from the meat. With heat treated meat particles, the indicator was totally inhibited but it was only partially inhibited by the unheated samples. Heat treatment of 100°C for 3 min was used in beef slurries made of tallow or muscle to recover pediocin AcH (class IIa bacteriocin produced by strains of *Pediococcus acidilactici*) added to the slurry as a crude preparation (Degnan and Luchansky, 1992). These authors indicated that heat treatment inactivated native or microbial protease activity and they obtained better recovery of pediocin with the heated than with the unheated slurries (Degnan and Luchansky, 1992). In studies of nisin added to meat, other treatments were used to release the protein-bound nisin such as boiling the samples under acidic conditions, but recovery of the added nisin remained poor (Bell and deLacy, 1986; Chung *et al.*, 1989).

Diluent used to inoculate the meat particles with bacteria influenced bacteriocin production *in situ*. This was not due to a lack of bacteriocin stability in the diluent because bacteriocin activity was still detected after 2 days of incubation at 25°C when the cell-free supernatant was diluted in 0.85% NaCl (Table 4-2). Composition of the growth medium was shown to influence bacteriocin production in lactic acid bacteria (Kozak and Dobrzanski, 1977). More precisely, the carbon source influences nisin production in *Lactococcus lactis* ssp. *lactis* (De Vuyst and Vandamme, 1992); and, peptone and Tween 80 were shown to promote bacteriocin production by *C. piscicola* LV61 and *Lactococcus lactis* ssp. *cremoris* J46, respectively (Schillinger *et al.*, 1993; Huot *et al.*, 1996). Immersion of the meat particles might influence the nutrient composition of the sample either by loss or acquisition of nutrients that promote bacteriocin production. The extent to which bacteriocin production is promoted by stimulating substances such as sucrose, peptone or Tween 80 in meat systems (e.g., processed meats) or in the diluent remains to be studied. Furthermore, alternative methods of inoculation need to be investigated. Embedding the inoculum in a gel or a gel-like substance containing the inoculum could be an interesting alternative while at the same time reducing the water loss due to evaporation on the carcass surface as is the case during the refrigeration process (Urbain and Campbell, 1987).

We postulated that the recovery of bacteriocin production by a non-producing culture when growing on a solid medium was due to the cells being exposed to a higher concentration of bacteriocin because the bacteriocin does not diffuse as readily as in liquid medium. The results from the meat experiment show that bacteriocin production occurred at low inoculum (Fig. 4-3 and Tables 4-4, 4-5 and 4-6) indicating that bacteriocin production in *C.*

piscicola LV17 was induced on the surface of meat in the same way that it was induced on a solid synthetic medium. However, bacteriocin production was better at 10^2 cfu/g than at 10^4 cfu/g in meat particles inoculated with *C. piscicola* LV17 (Tables 4-3 and 4-5). It is possible that a 10^2 cfu/g culture allows more bacteriocin to accumulate around the cells because the stationary phase will be reached later than with a 10^4 cfu/g culture. Induction would then be a function of the greater accumulation of bacteriocin from the basal level of expression around the cell because bacteriocin diffusion might vary with the type of solid medium used. Delayed and lower bacteriocin activity was detected with a 10^5 cfu/ml culture compared with the positive control at 10^7 cfu/ml, but bacteriocin activity was detected earlier and at a higher concentration in an induced culture at 10^4 cfu/ml compared with the 10^5 cfu/ml culture (Fig. 2-1, p. 91). What is important is that the threshold concentration of bacteriocin is reached before the stationary phase otherwise the maximum level of bacteriocin production is not achieved.

Bacteriocin activity on fat tissue from growth of *C. piscicola* LV17 (production *in situ*) or from absorption of bacteriocin at time zero using an inoculum containing bacteriocin (800 AU/ml; Table 4-6), was not as stable as the bacteriocin activity on meat particles (Table 4-5). With the pork fat tissue, bacteriocin activity is detected at a specific time (t = 1 day) and thereafter no activity is detected; whereas with meat particles, activity is still observed up to two days after the initial detection. These results suggest that enzymatic activity responsible for destruction of the bacteriocin in inoculated fresh meat may be more important than with the meat particle system. Degnan and Luchansky (1992) recovered 24 to 60% of the initial pediocin activity (non-encapsulated) added to beef slurry within 1.5 min of the addition of 30,000 AU/ml; after 35

min, recovery of 14 to 40% was obtained; and activity remained steady thereafter. They also observed that recovery from tallow was better than from a lean meat slurry. However, Bell and deLacy (1986) obtained different results with nisin. Nisin recovery was influenced by the fat content. As the fat content increased, the efficiency of recovery decreased. Nisin was recovered using an acid extract and it was not recovered as efficiently from meat as it was from other foods. After 28 days of storage at 5°C, Neilsen *et al.* (1990) demonstrated that pediocin on lean beef was stable and still active. Fang and Lin (1994) reported that nisin activity on cooked pork did not decrease as rapidly at 4°C as it did at 20°C indicating that storage conditions could affect the bacteriocin stability and efficiency in meat systems.

The results presented in Table 4-7 indicate that bacteriocin production at low inoculum ($\leq 10^4$ cfu/ml) can be induced at low temperature. Furthermore, bacteriocin production was detected on refrigerated vacuum packaged pork loin inoculated with *L. gelidum* UAL187 (McMullen *et al.*, 1995), but when the inoculated lean pork disks were incubated at room temperature limited bacteriocin production was observed (Table 4-6). This suggests that bacteriocin production on meat systems is initiated upon sensing a lower temperature and that it could be part of a cold shock response. The reduced growth rate may allow *C. piscicola* LV17 to produce enough bacteriocin from its basal level of expression to reach the necessary threshold before entering the stationary phase. Similar results were obtained with *Lactobacillus amylovorus* where greater bacteriocin production was observed at a low temperature that was unfavorable for growth of the organism (De Vuyst *et al.*, 1996).

Leisner *et al.* (1995) reported that the growth of *C. piscicola* LV17 on

lean beef under vacuum at 2°C is unpredictable. Ahn and Stiles (1990a) reported that *C. piscicola* LV17 grew at pH 5.6 on a synthetic medium but failed to produce bacteriocin. In this study, the pH of the lean tissue was 5.61 ± 0.06 whereas the fat was at pH 6.58 ± 0.12 which is a more suitable pH for bacteriocin production. Furthermore, growth of *C. piscicola* LV17 was better on fat than on lean suggesting that the conditions on fat tissue were more favorable. *Brochothrix thermosphacta* is an important spoilage organism of meat and meat products stored at refrigeration temperature under aerobic conditions. It spoils the meat by a "sour" off-odor. Under anaerobic conditions, growth of *B. thermosphacta* is inhibited at the normal pH of meat (Sulzbacher and Mclean, 1951; Egan and Grau, 1981). However, on high pH pork, designated dark, firm and dry (DFD), growth of *B. thermosphacta* is not impaired. *C. piscicola* LV17A (carnobacteriocin A producer) and LV17B (carnobacteriocins B2 and BM1 producer) inhibited several strains of *Brochothrix* when direct and deferred tests were performed on MRS agar (Gao, 1996). It remains to be seen if *C. piscicola* LV17 can inhibit this spoilage organism on DFD pork, because the pH will be more favorable for growth and bacteriocin production.

The pH of lean pork meat is limiting for bacteriocin production by *C. piscicola* LV17 whereas CMM is at pH 7 and APT is at pH 6.5 which more closely resemble the pH of certain types of processed meats (Buchanan, 1986). Consequently, *C. piscicola* LV17 might be better suited for use as a protective culture in processed meat systems than for preservation of raw meats. *C. piscicola* LV17 was isolated from refrigerated vacuum packaged pork and results for bacteriocin production at low incubation temperatures indicate that bacteriocin production can be induced with a low inoculum level of a non-

producing culture ($\leq 10^4$ cfu/ml). Bacteriocin production by *C. piscicola* LV17 is not a constitutive trait, but it can be induced under specific conditions and these conditions might identify a specific ecological niche. Generalizations regarding bacteriocin production cannot be made and each meat system of interest must be tested separately. More sensitive methods to detect bacteriocin in food systems must be developed to evaluate fully the potential of bacteriocinogenic strains as protective cultures in food.

4.5. References

Ahn, C. and Stiles, M.E. 1990a. Antibacterial activity of lactic acid bacteria isolated from vacuum-packaged meats. Journal of Applied Bacteriology 69:302-310.

Ahn, C. and Stiles, M.E. 1990b. Plasmid-associated bacteriocin production by a strain of *Carnobacterium piscicola* from meat. Applied and Environmental Microbiology 56:2503-2510.

Andersson, R. 1986. Inhibition of *Staphylococcus aureus* and spheroplasts of gram-negative bacteria by an antagonistic compound produced by a strain of *Lactobacillus plantarum*. International Journal of Food Microbiology 3:149-160

Barefoot, S.F. and Klaenhammer, T.R. 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Applied and Environmental Microbiology 45:1808-1815.

Bell, R.G. and deLacy, K.M. 1986. Factors influencing the determination of nisin in meat products. Journal of Food Technology 21:1-7.

Buchanan, R.L. 1986. Processed meats as a microbial environment. Food Technology 40(4):134, 136, 138.

Chung, K.T., Dickson, J.S. and Crouse, J.D. 1989. Effects of nisin on growth of bacteria attached to meat. Applied and Environmental Microbiology 55:1329-1333.

Daeschel, M.A. 1992. Procedures to detect antimicrobial activities of microorganisms. In: Ray, B. and Daeschel, M.A. (Ed.), Food biopreservatives of microbial origin. CRC Press, Inc., Boca Raton, FL, pp. 57-80.

Degnan, A.J. and Luchansky, J.B. 1992. Influence of beef tallow and muscle on the antlisterial activity of pediocin AcH and liposome-encapsulated pediocin AcH. Journal of Food Protection 55:552-554.

De Klerk, H.C. 1967. Bacteriocinogeny in *Lactobacillus fermenti.* Nature 214:609.

De Klerk, H.C. and Coetzee, J.N. 1961. Antibiosis among lactobacilli. Nature 192:340-341.

Delves-Broughton, J. 1990. Nisin and its uses as a food preservative. Food Technology 44(11):100, 102, 104, 106, 108, 111-112, 117.

De Vuyst, L. and Vandamme, E.J. 1992. Influence of the carbon source on nisin production in *Lactococcus lactis* subsp. *lactis* batch fermentations. Journal of General Microbiology 138:571-578.

De Vuyst, L., Callewaert, R. and Crabbé, K. 1996. Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. Microbiology (UK) 142:817-827.

Diep, D.B., Håvarstein, L.S. and Nes, I.F. 1995. A bacteriocin-like peptide induces bacteriocin synthesis in *Lactobacillus plantarum* C11. Molecular Microbiology 18:631-639.

Egan, A.F. and Grau, F.H. 1981. Environmental conditions and the role of *Brochothrix thermosphacta* in the spoilage of fresh and processed meat. In: Roberts, T.A., Hobbs, G., Christian, J.H.B. and Skovgaard, N. (Ed.), Psychrotrophic Microorganisms in Spoilage and Pathogenicity, Academic Press, Toronto, Canada.

Eijsink,V.G.H., Brurberg, M.B., Middelhoven, P.H. and Nes, I.F. 1996. Induction of bacteriocin production in *Lactobacillus sake* by a secreted peptide. Journal of Bacteriology 178:2232-2237.

Fang, T.J. and Lin, L.-W. 1994. Growth of *Listeria monocytegenes* and *Pseudomonas fragi* on cooked pork in a modified atmosphere packaging/nisin combination system. Journal of Food Protection 57:479-485.

Fields, F.O. 1996. Use of bacteriocins in food: regulatory considerations. Journal of Food Protection 59 suppl.:72-77.

Gao, Y. Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, AB. Personal communication.

Geis, A., Singh, J. and Teuber, M. 1983. Potential of lactic streptococci to produce bacteriocin. Applied and Environmental Microbiology 45:205-211.

Greer, G.G. and Jones, S.D.M. 1991. Effects of lactic acid and vacuum packaging on beef processed in a research abattoir. Canadian Institute of Food Science and Technology Journal 24:161-168.

Hoover, D.G. and Harlander, S.K. 1993. Screening methods for detecting bacteriocin activity. In: Hoover, D.G. and Steenson, L.R. (Ed.), Bacteriocins of lactic acid bacteria. Academic Press, Inc., San Diego, CA, pp. 23-39.

Huot, E., Barrena-Gonzalez, C. and Petitdemange, H. 1996. Tween 80 effect on bacteriocin synthesis by *Lactococcus lactis* subsp. *cremoris* J46. Letters in Applied Microbiology 22:307-310.

Jack, R.W., Tagg, J.R. and Ray, B. 1995. Bacteriocins of gram-positive bacteria. Microbiological Reviews 59:171-200.

Klaenhammer, T.R. 1988. Bacteriocins of lactic acid bacteria. Biochimie 70:337-349.

Klaenhammer, T.R. 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiological Reviews 12:39-85.

Kozak, W. and Dobrzanski, W.T. 1977. Growth requirements and the effect of organic components of the synthetic medium on the biosynthesis of the antibiotic nisin in *Streptococcus lactis* strain. Acta Microbiologica Polonica 26:361-368.

Leisner, J.J., Greer, G.G., Dilts, B.D. and Stiles, M.E. 1995. Effect of growth of selected lactic acid bacteria on storage life of beef stored under vacuum and in air. International Journal of Food Microbiology 26:231-243.

McMullen, L.M., Saucier, L., Leisner, J.J., Greer, G.G. and M.E. Stiles. 1995. Bacteriocinogenic activity of *Leuconostoc gelidum* UAL 187 in MAP fresh meat. Workshop on the Bacteriocins of Lactic Acid Bacteria: Applications and Fundamentals, Banff, P5.

Mørtvedt, C. and Nes, I.F. 1990. Plasmid associated bacteriocin production by a *Lactobacillus sake* strain. Journal of General Microbiology 136:1601-1607.

Nes, I.F., Diep, D.B., Håvarstein, L.S., Brurberg, M.B., Eijsink, V. and Holo, H. 1996. Biosynthesis of bacteriocins in lactic acid bacteria. Antonie van Leeuwenhoek 70:113-128.

Nielsen, J.W., Dickson, J.S. and Crouse, J.D. 1990. Use of a bacteriocin produced by *Pediococcus acidilactici* to inhibit *Listeria monocytogenes* associated with fresh meat. Applied and Environmental Microbiology 56:2142-2145.

Quadri, L.E.N., Sailer, M., Roy, K.L., Vederas, J.C. and Stiles, M.E. 1994. Chemical and genetic characterization of bacteriocins produced by *Carnobacterium piscicola* LV17B. The Journal of Biological Chemistry 269:12204-12211.

Rammelsberg, M. and Radler, F. 1990. Antibacterial polypeptides of *Lactobacillus* species. Journal of Applied Bacteriology 69:177-184.

Rhodehamel, E.J. 1992. FDA's concerns with sous vide processing. Food Technology 46(12):73-76.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989 Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Saucier, L., Poon, A. and Stiles, M.E. 1995. Induction of bacteriocin production in *Carnobacterium piscicola* LV17. Journal of Applied Bacteriology 78:684-690.

Schillinger, U., Stiles, M.E. and Holzapfel, W.H. 1993. Bacteriocin production by *Carnobacterium piscicola* LV61. International Journal of Food Microbiology 20:131-147.

Sulzbacher, W.L. and McLean, R.A. 1951. The bacterial flora of fresh pork sausage. Food Technology 5:7-8.

Tagg, J.R., Dajani, A.S. and Wannamaker, L.W. 1976. Bacteriocins of gram-positive bacteria. Bacteriological Reviews 40:722-756.

Urbain, W.M. and Campbell, J.F. 1987. Meat preservation. In: Price, J.F. and Schweigert, B.S. (Ed.), The science of meat and meat products, 3rd ed., Food and Nutrition Press, Inc., Wesport, CN, pp. 371-412.

West, C.A. and Warner, P.J. 1988. Plantacin B, a bacteriocin produced by *Lactobacillus plantarum* NCDO 1193. FEMS Microbiology Letters 49:163-165.

Worobo, R.W., Henkel, T., Sailer, M., Roy, K.L., Vederas, J.C. and Stiles, M.E. 1994. Characteristics and genetic determinant of a hydrophobic peptide bacteriocin, carnobacteriocin A, produced by *Carnobacterium piscicola* LV17A. Microbiology (UK) 140:517-526.

It's not so much what life gives us that matters, it's what we're doing with it!

Chapter 5

General Conclusions

C. piscicola LV17 was isolated from vacuum packaged pork. Why *C. piscicola* LV17 produces bacteriocin is not known, but it could be part of an evolutionary process that allows the organism to compete more efficiently with others and allows it to prevail in particular ecological niches. *C. piscicola* LV17 may have the potential to be used as a protective culture because its bacteriocins are detected early in the growth cycle when inoculated at 1% and its bacteriocins are active against strains of *Enterococcus* and *Listeria* (Ahn and Stiles, 1990b). However, when it was discovered that bacteriocin production was inoculum-dependent and that bacteriocin production only reached a detectable level in liquid medium when the inoculum is $\geq 10^6$ cfu/ml, the feasibility of using this bacteriocinogenic strain as a protective culture in food was questioned. If bacteriocin production is not constitutive and only occurs with a high inoculum on meat, the protective culture could spoil the meat before it exerts its beneficial competitive effect on the natural meat microflora. In *C. piscicola* LV17, three environmental factors were found to influence bacteriocin production: pH (Ahn and Stiles, 1990a), nutrients in the growth medium (Section 1.6.1, p. 46) and temperature (Table 4-7, p. 160) suggesting that bacteriocin production is regulated and that it can be induced.

The first question was, how can bacteriocin production be restored with a low inoculum ($\leq 10^4$ cfu/ml)? It was discovered that bacteriocin production can be induced at low inoculum by adding the cell-free supernatant (1%) of a carnobacteriocin-producing culture provided that it is added prior to the stationary phase of growth. The same result was obtained using purified bacteriocins or their sulfoxide derivatives, indicating that bacteriocin production is autoregulated. This was further confirmed by Northern and primer extension analyses (Fig. 3-1, p. 115 and 3-3, p. 119). Bacteriocin production in *C.*

piscicola LV17 is therefore tightly linked to the growth cycle of the producer strain. Induction can only occur if a specific ratio of the inducer (i.e., the bacteriocins or the sulfoxide derivatives) to cell concentration is reached before the stationary phase of growth (Fig. 2-1, p. 91). Bacteriocin positive phenotype can be recovered by growing the cells on a solid medium. The difference in bacteriocin production in a liquid vs. a solid medium suggests that the organism could have adapted its metabolism to a specific environment (i.e., meat). This was demonstrated in *Phosphobacterium fischeri* for production of luminescence in the light organ of certain fish and squid but not as a free living cell in sea water (Dunlup and Greenberg, 1991).

It was demonstrated by Quadri (1996) that a 10-kb *Pst*I fragment containing all of the open reading frames described in Fig. 1-2B, (p. 39), except *orf-β3,* were required for bacteriocin expression. However, based on the size of the transcript, the *cbnB2* transcript is long enough to include the two down-stream genes *cbiB2* and *orf-β3* (Chapter 3). Computer software was used to determine the possible function of the *orf-β3* gene. Four α-helices were postulated in *orf-β3* and a turn structure was found between amino acid residues 55 and 57 using the program PEPPLOT from GCG computer algorithms (Genetics Computer Group Inc., Deveraux *et al.,* 1984). However, none of the programs searched revealed a helix-turn-helix motif typical for a DNA binding protein domain (Voet and Voet, 1990). The protein encoded by *orf*-β3 has a theoretical pI of 4.61 and it is acidic in nature (Theoretical pI/MW for protein sequence, Swiss-Prot Data Bank, Appel *et al.,* 1994) which indicates that it is probably not a transmembrane protein as described by the program TMprep (Hofmann and Stoffel, 1993). A glycosylation site was predicted between amino acid residues 17 and 20 of *orf*-β3 with both PLOTSTRUCTURE

from GCG (Deveraux *et al.*, 1984) and PROSITE from Swiss-Prot (Appel *et al.*, 1994). Glycoproteins are believed to be involved in cell-cell recognition in mammalian cells (Voet and Voet, 1990). In the slime mold *Dictyostelium discoideum,* a glycoprotein referred to as a conditioned medium factor (CMF) is the signal for its cell density-sensing system. Although activity of CMF is reduced by deglycosylation, it is not part of the active site but it is involved in protein stability (Jain and Gomer, 1994). In prokaryotes, only a few examples of protein glycosylation have been reported. Apart from peptide linkage to the structural backbone of the cell wall, protein glycosylation has been described in *Neisseria gonorrhea* pilin (Parge et al., 1995) and S-layers in general (Küpcü et al., 1984). The exact function of *orf*-β3 remains to be elucidated as well as its role, if any, in the regulation system of carnobacteriocin production.

Bacteriocin production on meat particles and pork fat tissue at low inoculum indicated that these solid substrates induce bacteriocin production similar to agar media. It also indicated that bacteriocin induction probably occurs due to the fact that the inducer (i.e., the bacteriocin and its sulfoxide derivative) does not diffuse as readily in solid medium as it does in liquid medium. This allows more bacteriocin to be in contact with the producer cells and to have an autoregulatory effect. Fig. 5-1 is a schematic of a proposed model to explain how bacteriocin production may be induced by *C. piscicola* LV17. The model is based on several factors:

1. It is postulated that the accumulation of bacteriocin to the threshold level for induction of bacteriocin production occurs at the surface of the cell and it serves as the signal for bacteriocin production probably through the protein kinase and response regulator. By adding 100 AU/ml of partially purified

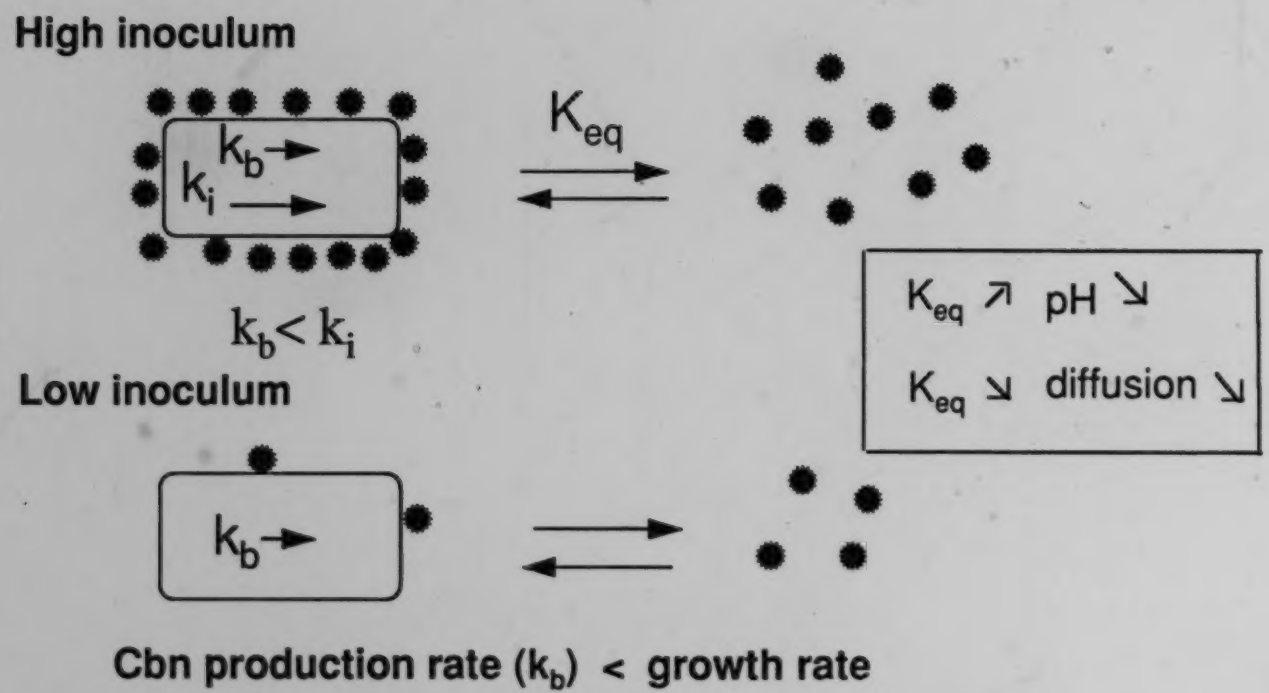


Fig. 5-1. Model for bacteriocin induction. For more details see general conclusions, p.177 Keq = equilibrium dissociation constant, k_b = rate constant of basal level of bacteriocin production, k_i = rate constant of bacteriocin production after induction, ● = carnobacteriocin.

bacteriocin to a suspension of 10^{10} cells per ml of phosphate buffer, Ahn and Stiles (1990b) demonstrated that carnobacteriocin was completely bound to the producer organism. The bacteriocin activity decreased to 67 AU/ml within 30 min of exposure at 25°C. However, other environmental signals can also affect bacteriocin production such as nutrients (source of yeast extract), pH (Ahn and Stiles, 1990a) and temperature (Table 4-7).

2. Constitutive production of bacteriocin proceeds at a slower rate (k_b = rate constant of basal level of bacteriocin production, independent of cell density) than the bacteriocin dissociation from the producer cell. After induction, bacteriocin production proceeds at a much faster rate (k_i = rate constant of bacteriocin production after induction, dependent on cell density and other environmental conditions) and more bacteriocin is released into the medium ($k_i > k_b$) and bacteriocin is detected in the supernatant. The "free and bound bacteriocin" reaction is defined by an equilibrium dissociation constant Keq (Keq = [free] / [bound]) and could be calculated using I^{125} radiolabelled pure bacteriocins. From there, the standard free energy ($\Delta G°$) can be calculated ($\Delta G = \Delta G° + R\,T\,\ln Keq$ at equilibrium $\Delta G = 0$, R is the gas constant and T is the temperature in °K). If $\Delta G°$ is negative this would mean that the proposed model is thermodynamically feasible (Voet and Voet, 1990).

3. Early in the growth cycle, the bacteriocin would have more affinity for the cell than for the growth medium concentrating the bacteriocin at the cell surface. This could account for bacteriocin induction at low inoculum when an exogenous source of bacteriocin is added to the growth medium. As the pH decreases during growth, bacteriocin is released from the cell (Hurst, 1981;

Yang *et al.*, 1992; Ra *et al.*, 1996) causing Keq to increase. By the time that the stationary phase is reached, there is not enough bacteriocin associated with the cell surface for bacteriocin induction to occur. Alternatively, *de novo* synthesis of bacteriocin could be stoped at low pH. Even if a source of bacteriocin is added during the stationary phase, no bacteriocin is detected. Furthermore, the Northern analysis (Fig. 3-1, p. 115) indicated that the basal level of expression is reduced when stationary phase is reached (Fig. 3-1, compare lanes 2, 3 and 4; p. 115). Is there a repressor induced during the stationary phase? None have been described so far but the system described here is "biphasic" in the sense that bacteriocin induction occurs prior to stationary phase and that attempts to induce bacteriocin production in stationary phase failed (Chapter 2).

4. On a solid surface, bacteriocin does not diffuse as readily as it does in liquid medium (Keq decreases when bacteriocin diffusion decreases). This would allow the bacteriocin to concentrate on the cell surface and to reach the threshold concentration for induction.

In summary, the rate of basal level of bacteriocin production is slower than the growth rate. At high inoculum, bacteriocin production is induced because more cells are present and this allows the threshold to be reached before the stationary phase.

The presence of an induction factor (IF), was described for *L. plantarum* C11 (PlnA) and *L. sake* LTH673 (Orf Y) by the research group of Dr. I.F. Nes in Norway (Diep *et al.*, 1995, 1996; Eijsink *et al.*, 1996). IF is described as a bacteriocin-like compound because it has a characteristic leader sequence of

the class II bacteriocin (Gly-Gly cleavage site) but it has no antimicrobial activity and it is smaller in size than most of the bacteriocins described so far. In this system, bacteriocin production is inoculum dependent but Bac$^+$ phenotype is not readily recovered by growth on solid medium unless IF is added to the medium. Similarly, with *C. piscicola* LV17, carnobacteriocin production is inoculum-dependent but Bac$^+$ phenotype can be recovered by growth on solid surface. Genes with homology to these IFs were subsequently identified in carnobacteriocin A and B2 operons (Quadri, 1996; Worobo, 1996). The induction of bacteriocin production by the synthetic peptide corresponding to *orf*-6 in the carnobacteriocin B2 operon indicates that it is most probably an induction factor homologous to those described in the sakacin P and plantaricin A operons (Quadri *et al.*, 1996). The difference between the two systems in recovery of Bac$^+$ phenotype on solid medium suggests that either the induction factor in *C. piscicola* LV17 is under a more "relaxed" regulation process or that other regulatory molecules are present. The results presented in this thesis indicate that the carnobacteriocins or their sulfoxide derivatives are also able to induce bacteriocin production. This was later confirmed using purified CbnB2 expressed as a recombinant protein in *E. coli* (Quadri *et al.*, unpublished). Gene disruption analyses of *orf*-6 and of the carnobacteriocin structural genes in conjunction with transcription analysis will help in understanding how these two regulatory elements act with respect to one another. At this time it is not known if induction of transcription is under the control of an activator or a repressor, but the presence of proteins with homology to the histidine kinase class of sensing and signaling pathways suggests that transcriptional activation is initiated by a response regulator similar to that suggested for nisin (Kuipers *et al.*, 1995). The induction of carnobacteriocin production using the synthetic IF (*orf*-6) in a transformant

which has its putative histidine kinase *cbnK* disrupted indicates that cross talk with other sensing systems is possible (Quadri *et al.*, unpublished).

The fact that some regulatory elements have been found does not mean that the whole regulatory puzzle has been solved. It is possible that we have only seen the "tip of the iceberg" of bacteriocin regulation. More than one cascade and level of regulation can be expected because several factors and conditions for bacteriocin production have been described. The regulation of antibiotic production and cell differentiation in *Streptomyces griseus* exemplify the level of "fine tuning" that is present for regulatory mechanisms in prokaryotic cells (Chater, 1989; Piepersberg, 1995). The signaling molecule, A-factor, is responsible for the "quorum sensing response" and a cascade of regulators respond to its induction.

As mentioned earlier, the pH of meat influences the capacity of *C. piscicola* LV17 to produce bacteriocin and makes it a better candidate for protective culture of processed meat systems than for fresh meat. However, when the pH of lean meat is high due to stress imposed on the animal before slaughter, bacteriocin production by *C. piscicola* LV17 could serve as a hurdle to control *Brochothrix* which grows better at a pH closer to 6 and which is sensitive to the carnobacteriocins produced by *C. piscicola* LV17 (Gao, 1996). It would be interesting to pursue the study of bacteriocin production at low temperatures and the cold shock response that might be associated with them. A temperature of 10°C represents an abusive storage temperature for commercial systems of refrigeration. Bacteriocin production at such a temperature could serve as an additional hurdle to spoilage for control of

pathogens and thereby assure the shelf life and the safety of the targeted food systems.

5.1. References

Ahn, C. and Stiles, M.E. 1990a. Antibacterial activity of lactic acid bacteria isolated from vacuum-packaged meats. Journal of Applied Bacteriology 69:302-310.

Ahn, C. and Stiles, M.E. 1990b. Plasmid-associated bacteriocin production by a strain of *Carnobacterium piscicola* from meat. Applied and Environmental Microbiology 56:2503-2510.

Appel, R.D., Bairoch, A. and Hochstrasser, D.F. 1994. A new generation of information retrieval tools for biologists: the example of the ExPASy WWW server. Trends in Biological Sciences 19:258-260.

Chater, K.F. 1989. Multiple level regulation of *Streptomyces* differentiation. Trends in Genetic 5:372-377.

Deveraux, J., Haeberli, P. and Smithies, O. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acid Research 12:387-395.

Diep, D.B., Håvarstein, L.S. and Nes, I.F. 1995. A bacteriocin-like peptide induces bacteriocin synthesis in *Lactobacillus plantarum* C11. Molecular Microbiology 18:631-639.

Diep, D.B., Håvarstein, L.S. and Nes, I.F. 1996. Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* C11. Journal of Bacteriology 178:4472-4483.

Dunlup, P.V. and Greenberg, E.P. 1991. Role of intercellular chemical communication in the *Vibrio fischeri* - monocentrid fish symbiosis. In: Dworkin, M. (Ed.), Microbial Cell-Cell Interactions. American Society for Microbiology, Washington, DC, pp. 220-253.

Eijsink, V.G.H., Brurberg, M.B., Middelhoven, P.H. and Nes, I.F. 1996. Induction of bacteriocin production in *Lactobacillus sake* by a secreted peptide. Journal of Bacteriology 178:2232-2237.

Gao, Y. Department of Agricultural, Food and Nutritional Sciences, personal communication.

Hofmann. K. and Stoffel, W. 1993. TMbase-A database of membrane spanning protein segments. Biological Chemistry Hoppe-Seyler 347:166.

Hurst, A. 1981. Nisin. Advances in Applied Microbiology 27:85-123.

Jain, R. and Gomer, R.H. 1994. A developmentally regulated cell surface receptor for a density-sensing factor in *Dictyostelium*. The Journal of Biological Chemistry 269:9128-9136.

Kuipers, O.P., Beerthuyzen, M.M., de Ruyter, P.G.G.A., Luesink, E.J. and de Vos, W.M. 1995. Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. The Journal of Biological Chemistry 270:27299-27304.

Küpcü, Z., März, L., Messner, P. and Sleytr, U.B. 1984. Evidence for the glycoprotein nature of the crystalline cell wall surface layer of *Bacillus stearothermophilus* strain NRS 2004/3a. FEBS Letters 173:185-190.

Parge, H.E., Forest, K.T., Hickey, M.J., Christensen, D.A., Getzoff, E.D. and Tainer, J.A. 1995. Structure of the fibre-forming protein pilin at 2.6 Å resolution. Nature 378:32-38.

Piepersberg, W. 1995. Streptomycin and related aminoglycosides. In: Vining, L.C. and Stuttard, C. (Ed.), Genetics and biochemistry of antibiotic production. Butterworth-Heinemann, Newton, MA, pp.531-570.

Quadri, L.E.N. 1996. Biochemical and genetic characterization of antimicrobial peptides produced by *Carnobacterium piscicola* LV17B. Ph.D. thesis, University of Alberta, Edmonton, AB.

Quadri, L.E.N., Stiles, M.E., Kleerebezem, M., Kuipers, O.P., and de Vos, W.M. 1996. Regulation of carnobacteriocins B2 and BM1 production by signal transduction. Fifth Synposium on Lactic Acid Bacteria. Veldhoven, The Netherlands, C2.

Quadri, L.E.N., Kleerebezem, M., Kuipers, O.P., de Vos, W.M., Roy, K.L., Vederas, J.C. and Stiles, M.E. Genes from *Carnobacterium piscicola* LV17B involved in bacteriocin production and immunity: evidence for global transcriptional regulation by different export peptides. Unpublished.

Ra, S.R., Qiao, M., Immonen, T., Pujana, I. and Saris P.E.J. 1996. Genes responsible for nisin synthesis, regulation and immunity form a regulon of two operons and are induced by nisin in *Lactococcus lactis* N8. Microbiology (UK) 142:1281-1288.

Voet, D. and Voet, J. 1990. Biochemistry. John Wiley and Sons, Inc., New York, NY.

Worobo, R.W. 1996. Characterization of two bacteriocins and a food grade plasmid from carnobacteria. Ph.D. thesis, University of Alberta, Edmonton, AB.

Yang, R., Johnson, M.C. and Ray, B. 1992. Novel method to extract large amounts of bacteriocins from lactic acid bacteria. Applied and Environmental Microbiology 58:3355-3359.